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Lines of Defined Genetic Constitution

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
In order to develop a better understanding of the molecular events that transform normal human prostate cells into prostate cancer, we have developed a system of cell transformation that permits the creation of immortalized and tumorigenic human prostate epithelial cell lines of defined genetic constitution. Expression of SV40 Large T antigen and hTERT, the catalytic subunit of telomerase, permitted immortalization. Transformation as assessed by the ability of these cells to form colonies in an anchorage independent fashion and to form tumors in immunodeficient host animals required the additional expression of an oncogenic version of the H-Ras protein. In addition, using hTERT alone, we have simultaneously created an immortalized human prostate stromal cell line. These cell lines provide an important foundation for future studies that will allow us to investigate the precise molecular interactions that lead to the development of prostate cancer. Ultimately, the elucidation of these critical molecular determinants of prostate cancer will permit the identification and confirmation of important targets for future therapeutic intervention.

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INTRODUCTION

Development of immortalized and tumorigenic prostate cell lines of defined genetic constitution
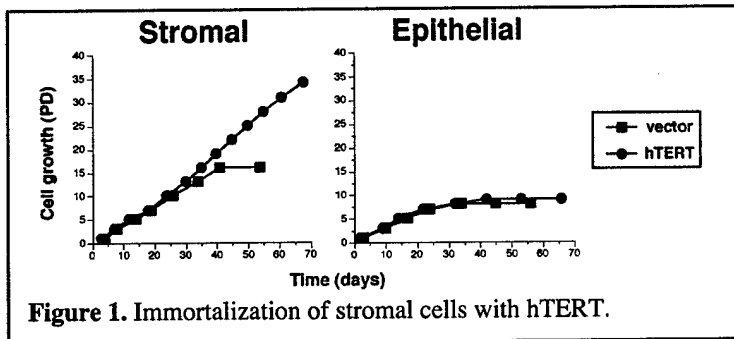
The study of prostate cancer cells derived from patients has elucidated many fundamental principles of malignant transformation and permitted the identification of several promising therapeutic targets. However, the critical changes that initiate cancer have been difficult to define with this approach. Currently efforts to understand the pathogenesis of prostate cancer are often limited by the difficulty in maintaining and propagating normal and malignant prostate epithelial cells (PrEC) *ex-vivo* as well as by disease and genetic heterogeneity. Specifically, the study of cancer cells derived from patients does not permit one to study the role of particular genes in the stepwise malignant conversion of normal PrEC to cancer cells in a defined genetic background. To address this need, we have developed a system of cell transformation that permits the creation of immortalized and tumorigenic human prostate epithelial cell lines of defined genetic constitution. Using these systems to define these molecular interactions that lead to the final phenotype of cancer will permit us to expand our understanding of prostate cancer and will identify important targets for therapeutic intervention.
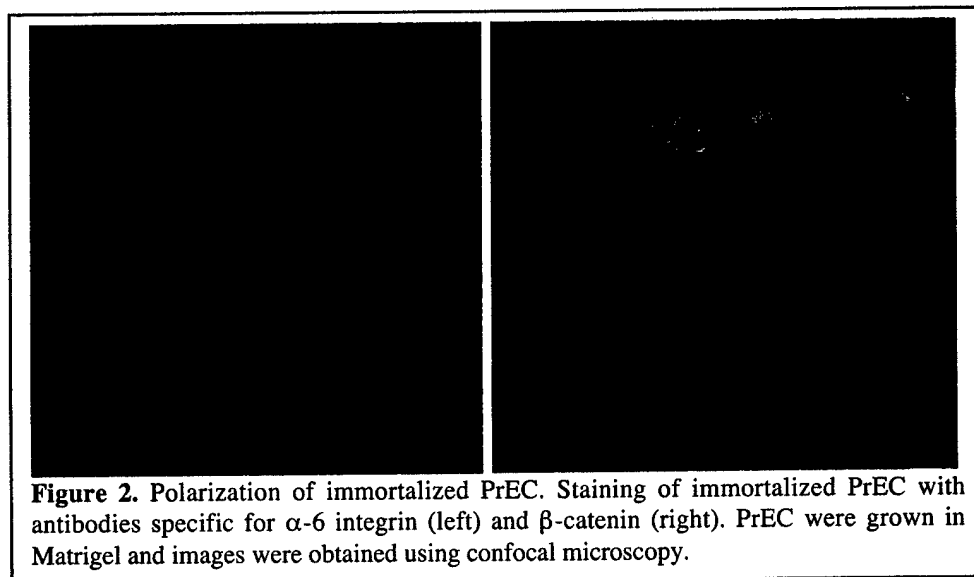
BODY

Task 1. Determine which genetic events cooperate with telomerase expression to immortalize prostate epithelial cells

In the first year of this award, we successfully immortalized prostate epithelial (PrEC) and stromal cells through the introduction of the SV40 large T antigen (LT) and the telomerase catalytic subunit hTERT. In the past year, we have confirmed that this immortalization requires the ablation of the p53 pathway since the introduction of dominantly interfering mutants of the retinoblastoma (pRB) pathway failed to allow immortalization even in combination with hTERT. The specific mutants we used included a cyclin dependent kinase 4 (CDK4) mutant unable to bind p16^{INK4A} and overexpression of cyclin D1. Thus, we have confirmed that ablation of the p53 pathway is necessary for the immortalization of prostate epithelial cells. Consistent with previously published work on fibroblasts (Bodnar et al., 1998), however, the expression of hTERT alone suffices to immortalize prostate stromal cells (Fig. 1).

Figure 1. Immortalization of stromal cells with hTERT.

Our initial characterization of these immortalized PrEC demonstrated that they express cytokeratins 5 and 14 as well as the basal cell epithelial marker p63. However, they fail to express the androgen receptor (AR). In the past year, we have characterized their phenotype more fully using confocal microscopy and three-dimensional culture systems as have been described for the propagation of mammary epithelial cells (Debnath et al., 2002; Weaver et al., 2002). Strikingly, we have found that these cells retain the ability to form polarized structures (Fig. 2). We plan to continue this work to characterize more fully the differentiation capacity of these immortalized cells.

Figure 2. Polarization of immortalized PrEC. Staining of immortalized PrEC with antibodies specific for α-6 integrin (left) and β-catenin (right). PrEC were grown in Matrigel and images were obtained using confocal microscopy.

Task 2. Determine which further events are required to convert immortalized cells into tumorigenic cells.

In the first year of funding, we introduced an oncogenic allele of the RAS oncoprotein into these immortalized PrEC and showed that this manipulation rendered the cells tumorigenic. However, since RAS mutations are rare in prostate cancer, we attempted to introduce activated alleles of Akt, a kinase target of phosphatidylinositol 3-kinase (PI3K), to mimic loss of PTEN, a tumor suppressor protein often mutated in prostate cancers (Ramaswamy and Sellers, 2000). Unfortunately, introduction of an activated allele of Akt failed to convert these immortalized PrEC into tumorigenic cells.
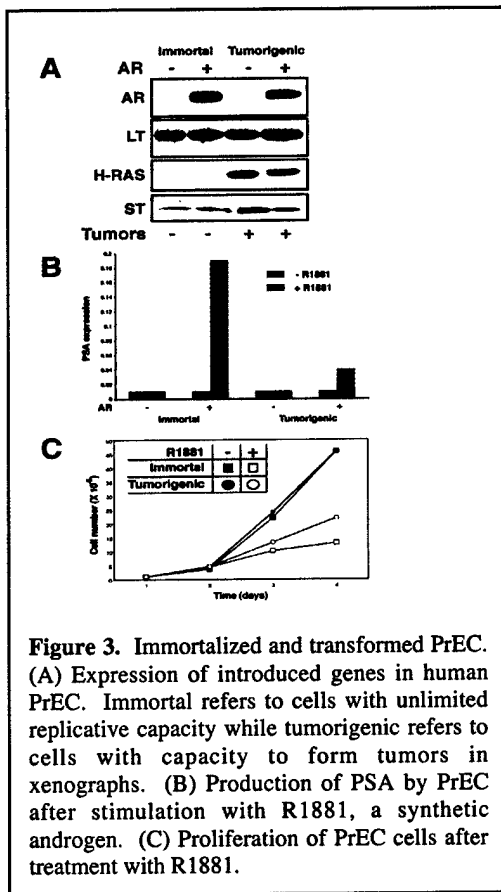
In order to develop experimental systems that more closely mimic human prostate cancers, we have begun to substitute the original genetic elements (LT, SV40 small T antigen (ST), hTERT, and RAS) used to transform these PrEC with genes associatedd with human prostate cancers. In initial experiments in mammary epithelial cells, a well-characterized cell type, we have found that an activated allele of PI3K and increased expression of c-myc will substitute for ST and RAS and permit the transformation of human mammary epithelial cells (Wei et al., 2003; Zhao et al., 2003) (See appendix). Moreover, activated alleles of Akt and Rac1 suffice to substitute for activated PI3K, explaining why activated alleles of Akt alone were unable to transform these cells. We are currently generating PrEC cells expressing this combination of genetic elements (LT, hTERT, activated PI3K and myc). Such cells will be characterized in the next year and will represent a further advance to develop tumorigenic PrEC that more closely resemble human cancers.

Task 3 Analyze immortalized and tumorigenic prostate epithelial and stromal cells *in vivo* to dissect critical stromal-epithelial interactions

While the experiments described above have and will result in increasingly useful experimental models of prostate cancer, we recognize that spontaneously arising prostate cancer is marked histologically by heterogeneity and the presence of significant stromal cells. Thus, we have begun to develop *in vitro* and *in vivo* experimental systems to study these interactions between tumor cells and stroma.

Our preliminary work indicates that the growth of immortalized PrEC in Matrigel facilitates epithelial cell polarization (Fig. 2). Over the past several months, we have begun to mix stromal cells with PrEC both in Matrigel cultures as well as in the kidney capsule in immunodeficient mice. At present, we have confirmed that tumorigenic PrEC retain the ability to form polarized structures; however, further characterization of the differentiation state and tumorigenic behavior of these models will require further experimentation in the third year of funding.

As noted above, these immortalized and tumorigenic PrEC lack AR. Since AR signaling plays a critical role in the development of prostate cancer, we have introduced AR into these cells and have determined the effects of androgen stimulation (Fig. 3). Incubation of these cells with the synthetic androgen induces PSA secretion and inhibits cell proliferation, which is partially offset by the expression of RAS. We will use these cells expressing AR in the *in vivo* and *in vitro* systems during this coming year.

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Figure 3. Immortalized and transformed PrEC. (A) Expression of introduced genes in human PrEC. Immortal refers to cells with unlimited replicative capacity while tumorigenic refers to cells with capacity to form tumors in xenographs. (B) Production of PSA by PrEC after stimulation with R1881, a synthetic androgen. (C) Proliferation of PrEC cells after treatment with R1881.

KEY RESEARCH ACCOMPLISHMENTS

1. Delineation of the role of p53 in the immortalization of PrEC
2. Development of three dimensional culture systems
3. Development of improved experimental systems to model prostate cancer
4. Development of an androgen-responsive PrEC line

REPORTABLE OUTCOMES

1. Publication of a manuscript delineating the role of PI3K signaling in cancer
2. Development of an androgen-responsive PrEC line

CONCLUSIONS

In the second year of this award, we have built upon the foundations established in year 1 and have development experimental prostate cancer models that increasingly mimic spontaneously arising human prostate cancers. In particular, the development of an androgen responsive cell line will faciliate that the study of this important pathway in prostate cancer pathogenesis.

Although these cell lines represent a significant advance, we will continue to improve these cell systems by substituting mutated alleles associated with prostate cancer and to develop three-dimensional culture systems to study these cells in more physiological environments. In addition, we will investigate the contribution of stroma to tumorigenicity in this system.

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Wei, W., Jobling, W. A., Chen, W., Hahn, W. C., and Sedivy, J. M. (2003). Abolition of cyclin-dependent kinase inhibitor p16Ink4a and p21Cip1/Waf1 functions permits Ras-induced anchorage-independent growth in telomerase-immortalized human fibroblasts. Mol Cell Biol *23*, 2859-2870.

Zhao, J., Gjoerup, O. V., Subramanian, R. R., Chen, W., Cheng, Y., Roberts, T. M., and Hahn, W. C. (2003). Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. In press.

APPENDIX

The following manuscripts are provided in support of this progress report:

Wei W, Jobling W, Chen W, Hahn WC, Sedivy JM. Abrogation of cyclin-dependent kinase inhibitors p16^{Ink4a} and p21$^{Cip1/Waf1}$ is sufficient for Ras-induced anchorage independent growth in telomerase-immortalized human fibroblasts. Mol. Cell Biol. 23: 2859-2870, 2003.

Zhao J, Gjoerup OV, Subramanian RR, Chen W, Cheng Y, Roberts TM, Hahn WC. Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. In press.

Abolition of Cyclin-Dependent Kinase Inhibitor p16^{Ink4a} and p21$^{Cip1/Waf1}$ Functions Permits Ras-Induced Anchorage-Independent Growth in Telomerase-Immortalized Human Fibroblasts

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Human cells are more resistant to both immortalization and malignant transformation than rodent cells. Recent studies have established the basic genetic requirements for the transformation of human cells, but much of this work relied on the expression of transforming proteins derived from DNA tumor viruses. We constructed an isogenic panel of human fibroblast cell lines using a combination of gene targeting and ectopic expression of dominantly acting mutants of cellular genes. Abolition of p21$^{Cip1/Waf1}$ and p16^{Ink4a} functions prevented oncogenically activated Ras from inducing growth arrest and was sufficient for limited anchorage-independent growth but not tumorigenesis. Deletion of the tumor suppressor p53 combined with abolition of p16^{Ink4a} function failed to mimic the introduction of simian virus 40 large T antigen, indicating that large T antigen may target additional cellular functions. Ha-Ras and Myc cooperated only to a limited extent, but in the absence of Ras, Myc cooperated strongly with the simian virus 40 small t antigen to elicit aggressive anchorage-independent growth. The experiments reported here further define specific components of human transformation pathways.

A state of irreversible growth arrest, commonly referred to as replicative senescence, has been documented in many normal human cells after a period of rapid proliferation in cell culture (20, 68). Since the proliferative period seems to be limited by the number of elapsed cell divisions, rather than chronological time, and indefinite proliferation (referred to as immortalization) depends on the accumulation of genetic lesions, it has been proposed that the senescence response may have evolved as a defense against the development of malignancy (8, 11). Indeed, most tumor cells bear mutations in the p53 and/or Rb pathways, both of which have been implicated in the establishment of replicative senescence (3, 60).

A number of significant differences have been documented between human and rodent cells in the regulation of the senescence response. Many rodent cell types either express telomerase or can spontaneously activate telomerase after a relatively limited culture period (46). Rodent cells are also more susceptible to malignant transformation. For example, normal mouse embryo fibroblasts are easily transformed by the combined expression of an activated oncogene, such as Ha-Ras^{G12V} (referred to hereafter simply as Ras), and an immortalizing function, such as Myc (29), adenovirus E1a (53), simian virus 40 (SV40) large T antigen (LT) (38), or human papillomavirus E6 or E7 (32, 45). These viral proteins all have the ability to interfere with the normal functions of the cellular p53 and/or retinoblastoma (Rb) proteins. The importance of

the p53 and Rb pathways in preventing tumor formation was further confirmed by mouse knockout studies, which showed that mouse embryo fibroblasts derived from p53$^{-/-}$ (22), p19 Arf$^{-/-}$ (24), or Rb/p107/p130$^{-/-}$ (55) animals could be transformed by activated Ras alone.

In contrast, both the senescence and transformation mechanisms are more stringently regulated in human cells (11, 56). The great majority of normal human cells do not express human telomerase (hTERT) activity (27), and immortalization is an extremely rare event. Likewise, Myc and Ras fail to transform primary human cells on their own (10, 11, 17, 81). More recent work has shown that Ras actually elicits a senescence-like arrest in both primary human and rodent cells (58). This somewhat unexpected finding can be viewed as yet another defense mechanism against inappropriate oncogenic signaling present in normal cells. In rodent cells, Ras-induced arrest can be eliminated by lesions in either the p53 or Rb pathways (58); however, in human cells, both pathways must be compromised (18, 44, 58, 75). Furthermore, bypassing Ras-induced arrest is not sufficient for full oncogenic transformation of human cells (18, 39, 44). Transformation of human foreskin fibroblasts, mammary epithelial cells, or keratinocytes has been shown to require the additional expression of SV40 small antigen (ST) (12, 18), which interferes with the function of protein phosphatase 2A (PP2A) (43, 80).

We have previously used gene targeting to knock out the p21 (6) and p53 (7) genes in normal, nonimmortalized human fibroblasts and used the resulting cell lines to study both replicative and induced senescence states. We presented data indicating that p53, p21, and Rb act sequentially and constitute the major pathway for establishing growth arrest in response to telomere attrition (75). p21 appears to be the major effector downstream of p53 responsible for both the establishment of

replicative senescence (6) and p14^{ARF}-induced premature senescence (75). In the studies reported here, we have expanded this genetic system by constructing additional isogenic cell lines to investigate the roles that p53, p21, and p16 play in premature senescence and transformation induced by oncogenic Ras. These experiments were stimulated by our desire to deduce clear-cut genetic functions for the steps required to convert a normal human cell into a malignant one. Most of the prior work in this area was predicated on the expression of viral transforming proteins, which are known to target multiple cellular proteins. We found that abolition of p21 and p16 functions was sufficient to bypass Ras-induced growth arrest and elicit limited anchorage-independent growth in response to Ras transformation. Loss of p53 gave the same results as loss of p21, confirming that p21 is a major p53 effector. Somewhat surprisingly, loss of p53 and p16 function did not mimic the introduction of LT in transformation elicited by the combination of Ras and ST, indicating that LT may target additional cellular function(s). In the presence of LT and ST, Myc cooperated to a limited extent with Ras to enhance anchorage-independent growth but did not further enhance in vivo tumorigenicity. Finally, in the absence of Ras, Myc cooperated with ST to elicit aggressive anchorage-independent growth, but the resulting cells did not form tumors in nude mice.

MATERIALS AND METHODS

Cell lines and culture conditions. LF1 is a normal human fibroblast cell strain derived from embryonic lung tissue (6). The p21$^{-/-}$ and p53$^{-/-}$ derivatives of LF1 have been described previously, as have their hTERT-immortalized derivatives (6, 7, 75). hTERT was introduced in all cases using retrovirus vector infection followed by drug selection. When originally derived, LF1/TERT cells were grown for more than 100 doublings past their calculated senescence point and were shown to express telomerase activity at the beginning and end of the passaging regimen. p21$^{-/-}$ and p53$^{-/-}$ cells were immortalized close to the end of their natural proliferative life span; introduction of empty vector did not yield any colonies, and all derived clones expressed telomerase activity. All three cell lines have been subjected to multiple rounds of drug selections, and in no case was loss of immortalized phenotype seen during genetic selection or subsequent passaging. All LF1 derivatives were cultured in Ham's F-10 medium supplemented with 15% fetal bovine serum, glutamine, penicillin, and streptomycin. Cultures were incubated at 37°C in an atmosphere of 93% N$_2$, 5% CO$_2$, and 2% O$_2$ (75, 76). The amphotropic Phoenix packaging cell line (70) was cultured at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum in an atmosphere of 5% CO$_2$ in air.

Retrovirus vectors. Retrovirus vectors of the pBabe series (40) and the pWZL-blasticidin (WZL-blast) retrovirus vector were obtained from J. Morgenstern (Millenium Pharmaceuticals). The pBabe-mEYFP vector was constructed by substituting the drug resistance gene in pBabe-puro with the membrane-bound enhanced yellow fluorescent protein (mEYFP) cDNA (Clontech). Ha-Ras^{G12V} cDNA was obtained from S. Lowe (Cold Spring Harbor) and subcloned into pBabe-mEYFP. hTERT, LT, and ST cDNAs (18, 37) were obtained from R. Weinberg (Whitehead Institute for Biomedical Research). The hTERT cDNA was subcloned into pBabe-puro, and SV40 ST cDNA was subcloned into pBabe-bleo. The cDNA encoding a Cdk4^{R24C}-cyclin D1 fusion protein (49) (DK) was obtained from R. N. Rao (Eli Lilly & Co.) and subcloned into the pWZL-blast, pBabe-puro, and pBabe-hygro vectors. Mouse c-Myc was tagged at the N terminus with a hemagglutinin (HA) tag and subcloned into the pWZL-blast and pBabe-bleo vectors. The amphotropic Phoenix packaging cell line (70) was obtained from G. Nolan (Stanford) and used according to the provided protocols (http://www.stanford.edu/group/nolan). The following drug concentrations were used for selection: hygromycin, 100 μg/ml; G418, 500 μg/ml; puromycin, 1 μg/ml; blasticidin, 3 μg/ml; and bleomycin, 500 μg/ml.

Immunoblotting. Immunoblotting analysis was performed as described previously (74, 75). Anti-p21 antibody C-19 (sc-397), anti-p53 antibody FL-393 (sc-6243), anti-Cdk4 antibody C-22 (sc-260), anti-LT+ST antibody Pab 108 (sc-148) were from Santa Cruz. Anti-c-Myc antibody 06-340 was from Upstate Biotechnology. Anti-HA tag antibody (MMS-101P) was from Covance. Anti-Ras antibody Ab-3 (OP40) and anti-Mdm-2 antibody Ab-1 (OP46) were from Calbiochem.

Soft-agar growth and mouse tumorigenicity assays. Soft-agar growth assays were performed as described previously (2). The infection efficiencies were determined prior to plating in soft agar by photographing random fields 48 h after infection under fluorescence and phase-contrast illumination and calculating the frequency of green cells as a percentage of total cells. At the time of plating in soft agar, cultures were trypsinized and counted, and 10^4 or 10^5 total cells were mixed with 1.5 ml of 0.4% Noble agar-DMEM (top layer) and then poured on top of 5 ml of solidified 0.8% Noble agar-DMEM (bottom layer) in 6-cm-diameter dishes. Cells were fed weekly by overlaying with 1.5 ml of fresh top layer solution. After 3 weeks, colonies were counted, and pictures were taken. Tumorigenicity assays were performed as described previously (17, 18) with minor modifications. A total of 3×10^6 cells were resuspended in 50 μl of phosphate-buffered saline, mixed with 50 μl of Matrigel solution, and immediately injected subcutaneously into nude mice. Each cell line was injected into four animals. Each animal was injected with Ras-infected cells in the right flank and with control cells infected with empty vector in the left flank. Female mice of the strain BALB/cAnNCrl-nuBR were obtained from Charles River at 8 weeks of age and injected within 1 week. Animals were not irradiated or otherwise treated.

Flow cytometry. Exponentially growing cells were trypsinized, fixed in ethanol (70% final concentration), and stored at 4°C as described previously (35). Immediately before use, cells were stained with propidium iodide and analyzed in a Becton-Dickinson FACSCalibur instrument.

RESULTS

Construction of cell lines. The starting objective of this study was to derive an isogenic set of cell lines in which to test the functional requirements for the p53 and Rb pathways (Fig. 1). The parental cell line was the LF1 lung fibroblast (6). Gene targeting was used to eliminate the function of the p21 and p53 genes (6, 7). To phenocopy loss of function of p16, we expressed the R24C mutant of Cdk4 which does not bind p16 and is thus insensitive to its inhibitory effects (78). This mutant has been frequently used to enforce p16-insensitive Cdk4 activity (18, 41, 48, 50, 58, 65) and is most effective when coexpressed with cyclin D. Since the scope of our genetic manipulations was limited by the relatively small number of dominant selectable markers, we chose to express a fusion protein between Cdk4^{R24C} and cyclin D1 (DK) (28, 49). This strategy allows the stoichiometric expression of Cdk4^{R24C} and cyclin D1 from a single retrovirus vector. Expression of DK in LF1 cells elicited a limited extension of life span (approximately five population doublings [data not shown]) at the end of which the cultures entered into a typical senescence (M1)-like state, very similar to the findings of Morris and coworkers (41) who used Cdk4^{R24C}. Furthermore, LF1/DK cells were immune to the inhibitory effects of p16 expression (Fig. 2). These findings indicate that the expressed DK protein has biological activity, and that, as expected, it renders cells resistant to the inhibitory effects of elevated p16 expression.

The pedigrees for all cell lines are shown in Fig. 1, and the retrovirus vectors used in their construction are summarized in Table 1. In all cases of immortalization with hTERT, cell lines were tested for telomerase activity using the telomere repeat amplification protocol assay (25, 74) and subsequently passaged extensively to verify the immortalized phenotype. Expression of the relevant proteins was demonstrated in all cell lines by immunoblotting (Fig. 3). Proliferation was measured during exponential growth phase using standard growth curves; the doubling times are shown in Table 1. The expression of DK in all cases accelerated proliferation. The expression of LT, ST,
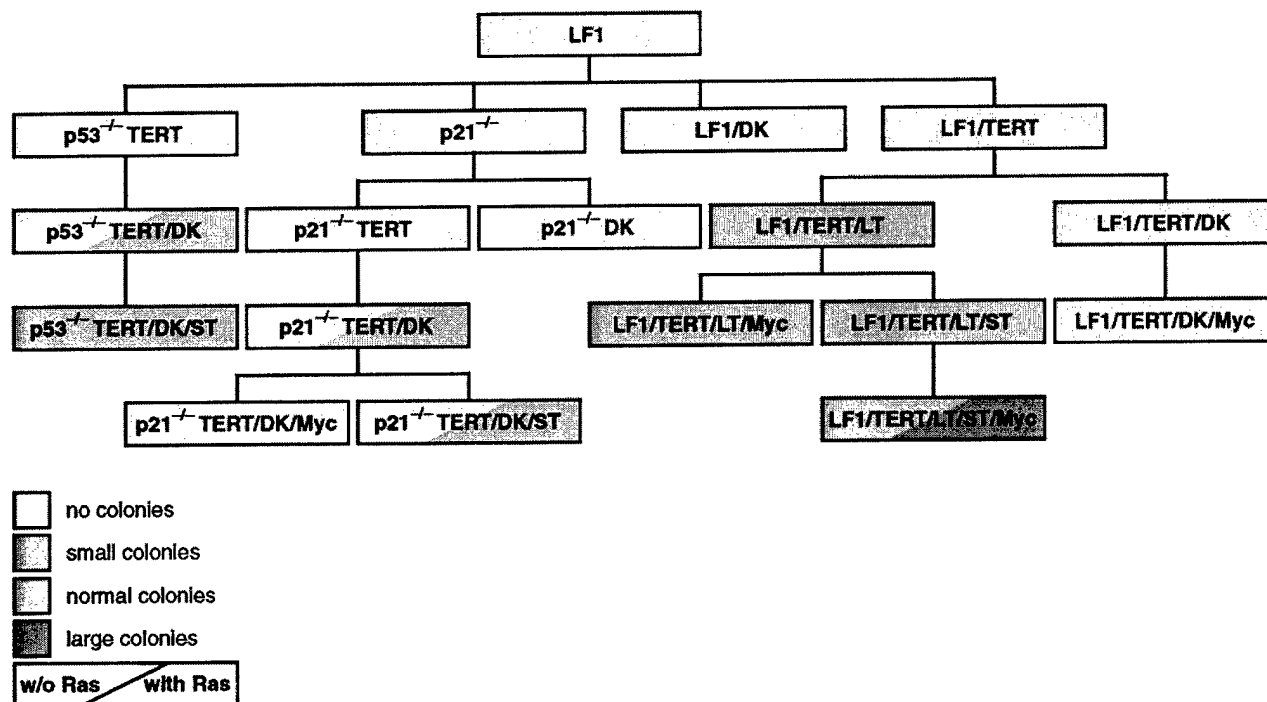
FIG. 1. Schematic representation of cell line pedigrees and anchorage-independent growth phenotypes. Primary data for soft-agar colony formation are shown in Fig. 5 and Table 2. Details of cell line construction are shown in Table 1. Colony sizes were defined as follows (see also Fig. 5). Small colonies were multicellular aggregates estimated to contain 30 to 100 cells. These colonies were visible only microscopically and were estimated to be 0.1 mm or less in diameter. Normal colonies were macroscopically visible colonies estimated to be between 0.2 and 1.0 mm in diameter. Large colonies were macroscopically visible colonies estimated to be >1.0 mm in diameter.

and Myc, in various combinations, also enhanced proliferation. The fastest growing cell line, LF1/TERT/LT/ST/Myc (doubling time of 20 h) was accelerated more than twofold relative to the LF1/TERT cell line (doubling time of 43 h) from which it was derived.

Ras does not induce growth arrest in p21$^{-/-}$TERT/DK cells. Overexpression of Ras in normal human fibroblasts results in a senescence-like state (58), and immortalization with hTERT does not abolish this response (74). Ras failed to induce premature senescence in cells in which both the p53 and Rb pathways are inactivated by E1a (58), E6 plus E7 (39), or LT (18). Ras was, however, able to induce premature senescence in human fibroblasts with disrupted p21 or p53 genes (75), as well as in human fibroblasts coexpressing the R24C mutant of Cdk4 and cyclin D1 (58). Furthermore, LT mutants unable to interact with either Rb or p53 were unable to protect human fibroblasts from Ras-induced arrest (18). In agreement, in our hands, LF1 fibroblasts expressing only the DK fusion protein displayed a clear premature senescence response after infection with a Ras-expressing retrovirus vector (data not shown).

To investigate the effects of abolition of both p21 and p16 function, p21$^{-/-}$TERT/DK cells were infected with the pBabe-mEYFP/Ras retrovirus vector (or empty vector control). Seven days after infection, the cultures were harvested, fixed, stained with propidium iodide, and analyzed by two-parameter flow cytometry for mEYFP expression and cell cycle distribution (Fig. 4). As expected, in p21$^{-/-}$TERT cells not expressing DK, Ras reduced the fraction of mEYFP-positive cells (Fig. 4E and F), indicating that the cells were at a proliferative disadvan-

tage. Furthermore, the mEYFP-positive cells displayed a cell cycle distribution indicative of growth arrest, namely, an increase in G_0/G_1 fractions and a decrease in S and G_2 fractions (Fig. 4I and J). The approximately threefold decrease in S-phase content is especially noteworthy. In contrast, infection of p21$^{-/-}$TERT/DK cells with the pBabe-mEYFP/Ras retrovirus vector did not elicit any signs of cell cycle arrest (Fig. 4G, H, K, and L); in fact, S-phase content was significantly increased in response to Ras (Fig. 4K and L). p53$^{-/-}$ cells behaved in a fashion similar to that of p21$^{-/-}$ cells: Ras elicited cell cycle arrest in the absence of DK (Fig. 4A and B) but not in the presence of DK (Fig. 4C and D). Unfortunately, the p53$^{-/-}$TERT/DK cell line became tetraploid in the course of these experiments, which complicated the subsequent cell cycle analysis. Taken together, our results are in agreement with most previous studies indicating that interference with both p53 and pRb pathways is necessary to avoid Ras-induced growth arrest. Furthermore, we have pinpointed p21 as the critical downstream effector of p53 and have established the minimal sufficient intervention as the joint abolition of p21 and p16 function.

Losses of p21 and p16 function are the minimum requirements for anchorage-independent growth in response to Ras. Having established the requirements to bypass the proliferative inhibition by Ras, we proceeded to investigate the minimum requirements to transform human fibroblasts to anchorage-independent growth. Since many of the cell lines are multiply marked with drug resistance markers and to achieve maximum consistency among the different cell lines, Ras was
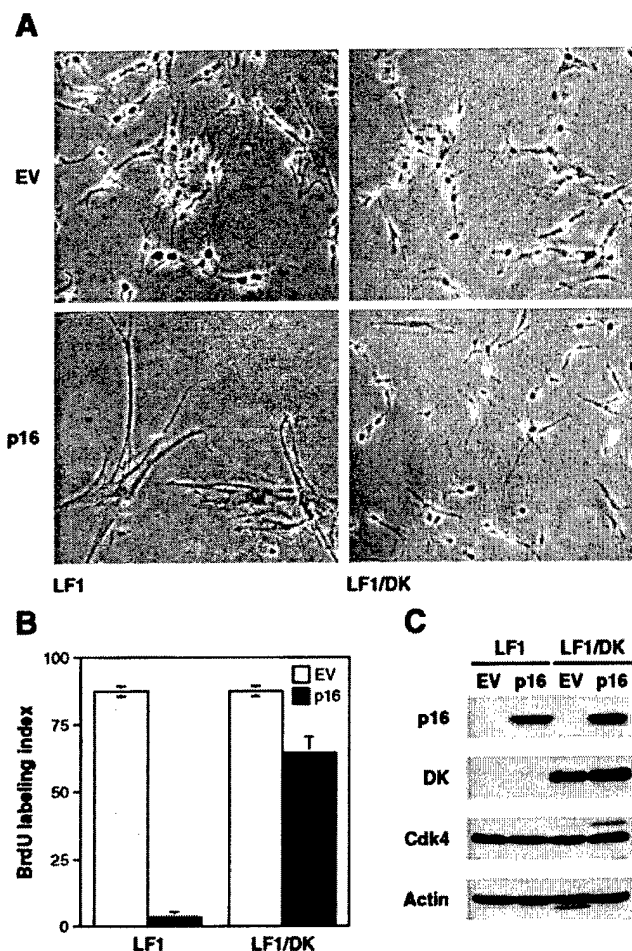
FIG. 2. DK-expressing cells are resistant to p16-induced premature senescence. (A) Photomicrographs of LF1 and LF1/DK cells infected with p16-expressing retrovirus. Cells were infected with pBabe-puro/Ras and pBabe-puro empty vector (EV) viruses. Pictures were taken 5 days after the start of puromycin selection. (B) Bromodeoxyuridine (BrdU) incorporation assays. Virus-infected and puromycin-selected cultures were labeled for 48 h with BrdU. Following immunohistochemical staining, total and BrdU-positive nuclei were counted in random fields. (C) Expression of p16 and DK proteins. Virus-infected and puromycin-selected cultures were harvested, and immunoblots were probed with the indicated antibodies. Cdk4 and DK proteins were both visualized by using an anti-Cdk4 antibody.

introduced using the pBabe-mEYFP retrovirus vector (empty pBabe-mEYFP vector was used as the control in all cases). Two days after infection, the cultures were observed under a fluorescence microscope to assess the efficiency of infection. Seven days after infection, the cultures were harvested by trypsinization and plated in soft agar. Aliquots were also processed for immunoblotting to ascertain the expression of the Ras protein, as well as all the other relevant proteins in each cell line (Fig. 3). Soft-agar plates were photographed at 3 weeks, and the incubation was continued, with regular feeding, for up to 5 weeks. Representative photomicrographs of the soft-agar plates are shown in Fig. 5, quantitative data are presented in Table 2, and the data are summarized in Fig. 1. Finally, all Ras-infected pools were injected into nude mice to determine in vivo tumorigenicity (Table 3).

Cell lines LF1, LF1/DK, LF1/TERT, LF1/TERT/DK, p21$^{-/-}$, p21$^{-/-}$TERT, and p53$^{-/-}$TERT did not form colonies in soft agar either with or without Ras infection, even after 5 weeks of incubation, consistent with the observation that the growth of all these cell lines is inhibited by Ras. p21$^{-/-}$TERT/DK cells infected with Ras formed small but clearly delineated colonies after 3 weeks of incubation (control vector-infected cells did not form colonies up to 5 weeks). The colonies were densely packed with cells, and after picking expansion with glass capillaries and expanded, the cells could be replated in soft agar with similar plating efficiencies. p21$^{-/-}$TERT/DK cells infected with Ras did not form tumors in nude mice up to 6 months after injection. Furthermore, a cell line established from a soft-agar colony that replated well in soft agar did not form tumors in vivo. Nonimmortalized p21$^{-/-}$DK cells did not produce colonies in soft agar after infection with Ras; this failure is explained by the fact that due to the multiple genetic interventions, these cultures were near the end of their proliferative life span. p53$^{-/-}$TERT/DK cells were not transformed by Ras to any greater extent than p21$^{-/-}$TERT/DK cells, either in the soft-agar or nude mouse assays. Thus, by both assays, the loss of p21 or p53 produces very similar end points. We conclude that a minimum of two clearly delineated genetic alterations are required by Ras to elicit anchorage-independent growth: loss of p21 and p16 functions.

Loss of p53 and p16 function is not equivalent to expression of LT. Since it has recently been demonstrated that ST is required in addition to LT for full transformation of human fibroblasts by Ras (18, 81), we introduced ST into p53$^{-/-}$TERT/DK and p21$^{-/-}$TERT/DK cells and repeated the Ras transformation assays. Surprisingly, although both the soft-agar plating efficiency and colony size were somewhat improved, transformation by Ras clearly did not reach the level of robustness elicited by the combination of LT and ST. Furthermore, expression of Ras in p53$^{-/-}$TERT/DK/ST and p21$^{-/-}$TERT/DK/ST cells did not result in tumor formation in nude mice, up to 6 months after injection into the animals (Table 3). Expression of ST allowed p53$^{-/-}$TERT/DK but not p21$^{-/-}$TERT/DK cells to form small colonies in soft agar even without Ras, but the introduction of Ras did not further enhance growth in soft agar.

A number of factors may have contributed to the observed small colony size in soft agar and lack of tumor formation in vivo by p53$^{-/-}$TERT/DK/ST/Ras cells. First, the expression of Ras by the pBabe-mEYFP vector could be insufficient, despite the fact that very good expression (Fig. 3) was documented by immunoblotting the pools of cells at the time of plating or injection. Second, the LF1 strain of human lung fibroblasts could be more resistant to transformation than the BJ strain used in prior experiments (17, 18). Third, the conditions of soft-agar plating and especially the mouse tumorigenicity assays could be sufficiently different from those used previously. To address these issues, we performed a number of control experiments. First, TERT, LT, and ST retroviruses were introduced into LF1 fibroblasts to derive a series of cell lines equivalent to those previously constructed in the BJ fibroblast background (17, 18). The appropriate expression of LT and ST proteins was verified by immunoblotting (Fig. 3). Second, BJ fibroblasts expressing TERT, LT, ST, and Ras (BJELR) were

TABLE 1. Construction and proliferation rates of cell lines

Cell line	Construction of cell line	Doubling time (h)[a]
LF1	No virus	34[b]
LF1/TERT	LF1 plus pBabe-puro/hTERT (clonal)	43
LF1/TERT/DK	LF1/TERT plus pWZL-blast/DK (clonal)	43
LF1/TERT/DK/Myc	LF1/TERT/DK plus pBabe-bleo/Myc (pooled)	43
LF1/TERT/LT	LF1/TERT plus Neo-LT (pooled)	43
LF1/TERT/LT/Myc	LF1/TERT/LT plus pWZL-blast/Myc (pooled)	25
LF1/TERT/LT/ST	LF1/TERT/LT plus pBabe-hygro/ST (pooled)	27
LF1/TERT/LT/ST/Myc	LF1/TERT/LT/ST plus pWZL-blast/Myc (pooled)	20
$p21^{-/-}$	No virus	48[c]
$p21^{-/-}$TERT	$p21^{-/-}$ plus pBabe-puro/hTERT (pooled)	66
$p21^{-/-}$TERT/DK	$p21^{-/-}$TERT plus pWZL-blast/DK (clonal)	60
$p21^{-/-}$TERT/DK/ST	$p21^{-/-}$TERT/DK plus pBabe-bleo/ST (pooled)	60
$p21^{-/-}$TERT/DK/Myc	$p21^{-/-}$TERT/DK plus pBabe-bleo/Myc (pooled)	30
$p53^{-/-}$TERT	$p53^{-/-}$ plus pBabe-puro/hTERT (clonal)	74
$p53^{-/-}$TERT/DK	$p53^{-/-}$TERT plus pBabe-hygro/DK (pooled)	43
$p53^{-/-}$TERT/DK/ST	$p53^{-/-}$TERT/DK plus pBabe-bleo/ST (pooled)	43

[a] The variance associated with the doubling times was within 5 to 10% of the mean doubling time given for each cell line.
[b] Growth of nonimmortalized LF1 cells was determined at mid passage.
[c] Growth of nonimmortalized $p21^{-/-}$ cells was determined at late passage.

used as controls in side-by-side soft-agar and nude mouse assays. LF1/TERT/LT/ST cells were transformed efficiently by the pBabe-mEYFP/Ras vector to anchorage-independent growth (Fig. 5), and the size range of the soft-agar colonies was comparable to that shown by BJELR cells (data not shown). Thus, it appears that the pBabe-mEYFP/Ras vector is competent to transform LF1/TERT fibroblasts if coexpressed with LT and ST. Furthermore, both LF1/TERT/LT/ST/Ras and BJELR cells produced in vivo tumors in our hands (Table 3). Therefore, the most reasonable explanation for the failure of Ras to fully transform $p53^{-/-}$TERT/DK cells is that the expression of LT elicits (or abolishes) a cellular response that goes beyond the loss of p53 and p16 function.

Myc cooperates with ST to promote strong anchorage-independent growth but not in vivo transformation. Since c-Myc has been well documented to collaborate with Ras in the transformation of rodent cells (29), it was of interest to introduce ectopic c-Myc expression into our isogenic panel of cell lines. In most cases, c-Myc was a strong growth-promoting agent, shortening exponential-phase doubling times by as much as twofold (Table 1). The one exception was the LF1/TERT/DK/Myc cell line; however, these cells expressed only low levels of Myc (Fig. 3, lanes 29 and 30), possibly due to the induction of apoptosis by high ectopic Myc expression. In contrast, $p21^{-/-}$TERT/DK/Myc cells displayed easily demonstrable ectopic Myc expression (Fig. 3, lanes 21 and 22), significantly accelerated proliferation, and a distinct small and compact cell shape. However, when deprived of anchorage, $p21^{-/-}$TERT/DK/Myc cells both in the absence and presence of Ras underwent apoptosis. As expected, expression of LT protected cells from Myc-induced apoptosis, and ectopic Myc expression significantly augmented the proliferation of LF1/TERT/LT cells, both in the presence (Table 1) and absence (Fig. 5) of anchorage. Surprisingly, Myc did not cooperate with Ras under these conditions, and LF1/TERT/LT/Myc cells formed only small colonies in soft agar both in the absence and presence of Ras (Fig. 5).

Perhaps the most surprising result was the promotion of strong anchorage-independent growth by the introduction of Myc into the LF1/TERT/LT/ST cell line (Fig. 5). This effect occurred in the absence of Ras and was thus the result of cooperation between Myc and ST. Exponential-phase proliferation rates were the highest of all cell lines in the panel (20 h [Table 1]), an increase of more than twofold relative to the parental LF1/TERT cell line (43 h). Both the plating efficiency and colony size in soft agar were significantly enhanced and equivalent to that elicited by Ras in LF1/TERT/LT/ST cells. However, in stark contrast to LF1/TERT/LT/ST/Ras cells, which were highly tumorigenic in vivo, LF1/TERT/LT/ST/Myc cells showed only negative results by the nude mouse assay (Table 3). Introduction of Ras into LF1/TERT/LT/ST/Myc cells further enhanced colony size in soft agar but did not increase the in vivo tumorigenicity beyond that seen with LF1/TERT/LT/ST/Ras cells.

DISCUSSION

It has been known for quite some time that human cells are much more resistant to both immortalization and malignant transformation than their rodent counterparts (56). However, other than sweeping generalizations that it takes more genetic lesions to fully transform a human cell, the mechanistic underpinnings of this observation have remained elusive. Only recently has complete transformation of normal human cells been achieved with defined genetic interventions (17, 18, 34, 51, 81), namely, the expression of hTERT, SV40 LT, SV40 ST, and Ras. However, since some of these interventions entailed the expression of DNA tumor virus oncoproteins, the corresponding list of equivalent cellular functions is still not completely understood. This is because the viral oncoproteins tar-
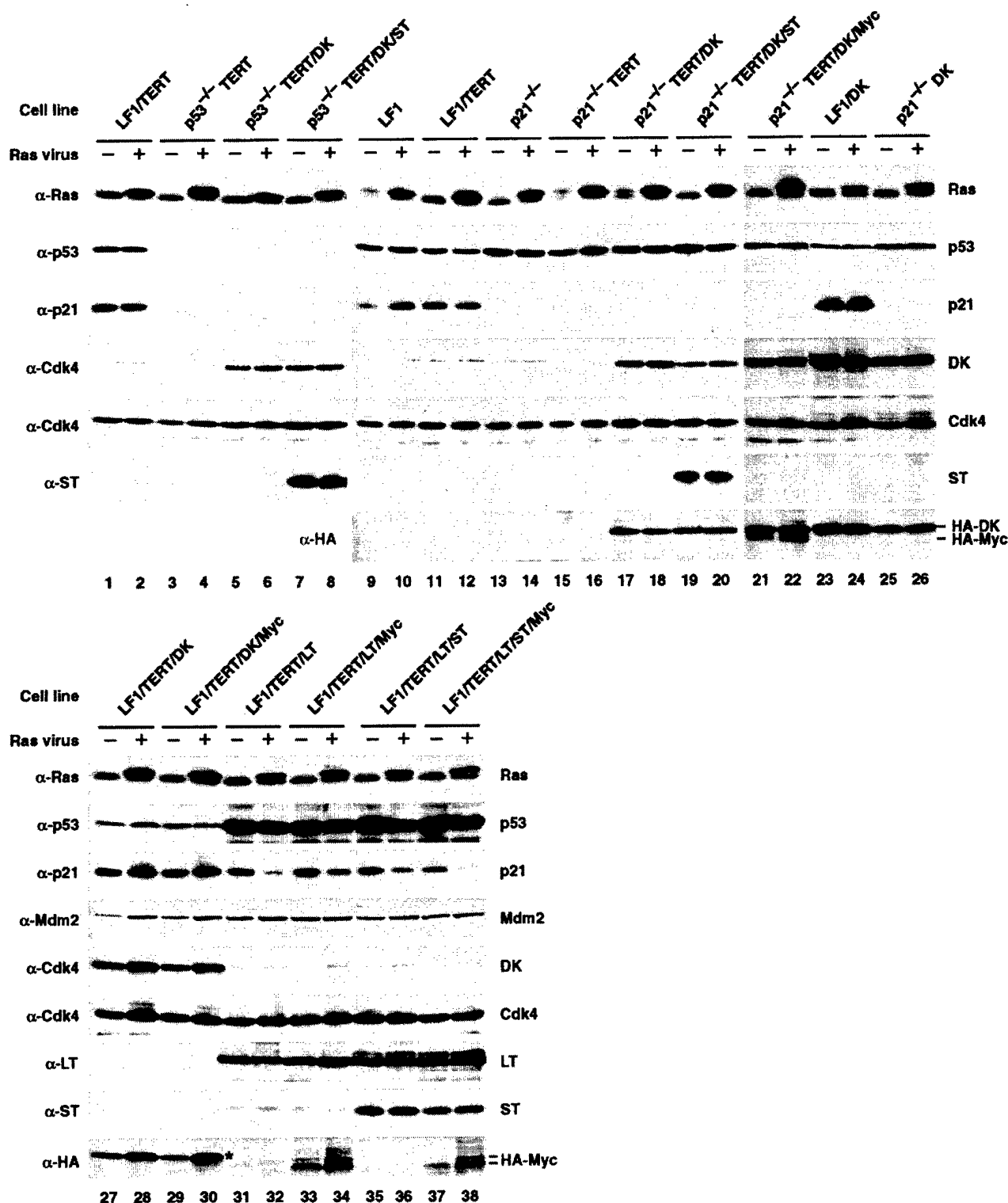
FIG. 3. Immunoblot analysis of Ras-infected pools of cells. Exponentially growing cultures were infected with pBabe-mEYFP/Ras or empty pBabe-mEYFP vectors. Cultures were not subjected to selection with any drugs and were kept in the exponential growth phase by subculturing as needed. Six days after infection, four 10-cm-diameter dishes were harvested, pooled, and processed for immunoblotting. The cell line is indicated above each pair of lanes, and the virus used to infect the cells is indicated above each lane as follows: −, empty vector; +, Ras vector. The antibodies used (anti-Ras [α-Ras], etc.) are indicated to the left of the gels. The proteins detected are indicated to the right of the gels. DK, which has a HA tag, can be detected with both anti-Cdk4 and anti-HA antibodies. HA-tagged Myc (HA-Myc) was detected with anti-HA antibody and migrates as a doublet (lanes 33, 34, 37, and 38). The upper band of this doublet comigrates with the DK protein, which is also detected by the HA antibody (lanes 21 and 22). Note the relatively low expression of Myc in the LF1/TERT/DK/Myc cell line (lanes 29 and 30) (the position of the DK protein is marked by an asterisk), whereas the Myc protein is easily seen in the p21^{−/−}TERT/DK/Myc cell line (lanes 21 and 22).
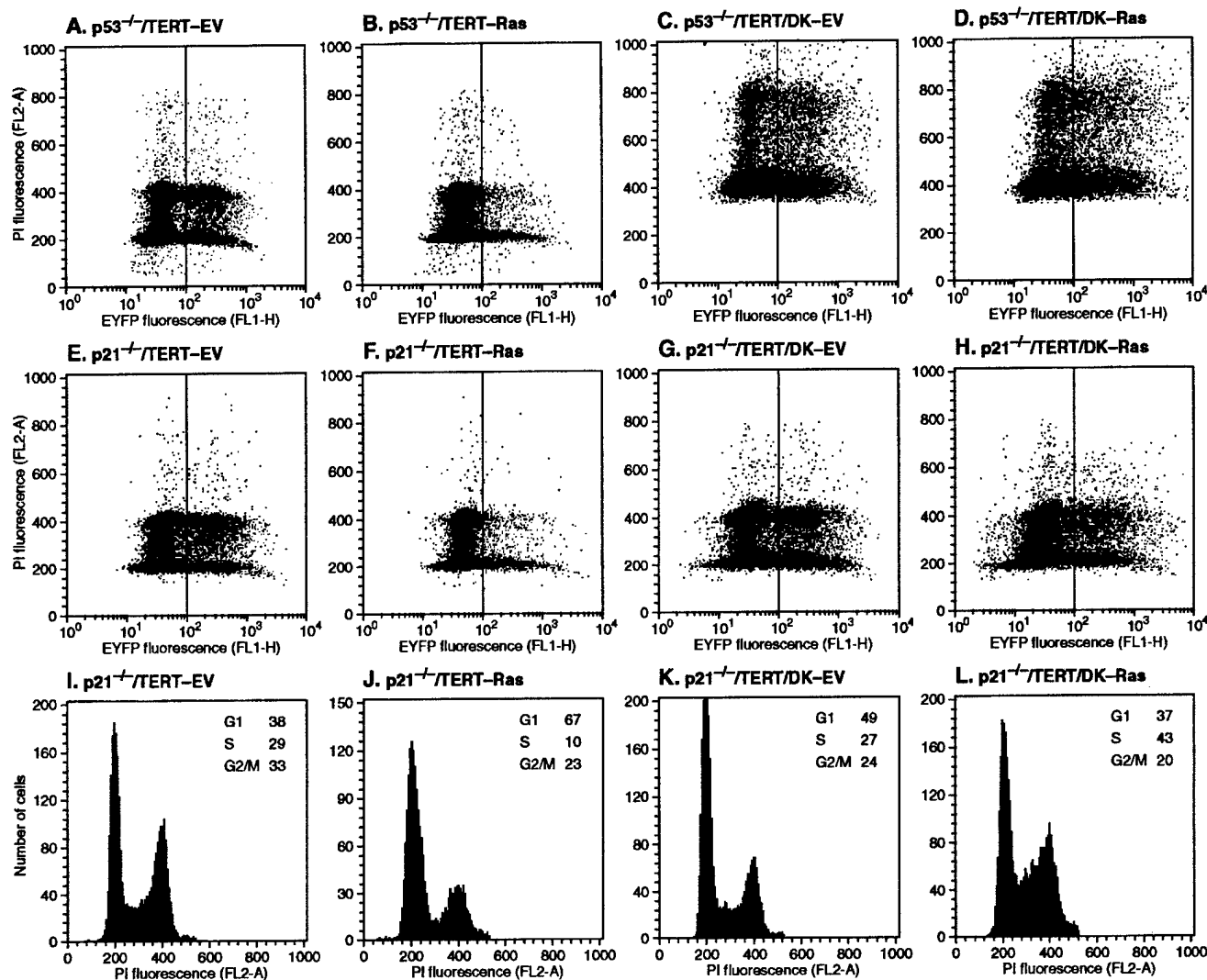
FIG. 4. Ras expression does not inhibit the proliferation of p53$^{-/-}$TERT/DK and p21$^{-/-}$TERT/DK cells. The cell lines were infected and propagated as described in the legend to Fig. 3. Seven days after infection, cells were harvested, fixed with 70% ethanol, stained with propidium iodide (PI), and analyzed by two-parameter flow cytometry. (A to H) Dot plots of PI and mEYFP fluorescence (aggregates were gated out on the forward scatter/side scatter dot plot). (I to L) PI fluorescence histograms of green (high mEYFP fluorescence) cells from panels E to H. EV, empty virus (pBabe-mEYFP).

get multiple cellular effectors, some of which remain poorly defined or even unknown. We demonstrate here that a homozygous knockout of p21 combined with the expression of a p16-insensitive Cdk4-cyclin D1 fusion protein suffices to overcome the arrest induced by Ras. This finding is consistent with previous studies utilizing viral oncoproteins that demonstrated a need to interfere with both the p53 and Rb pathways in human cells (14, 18, 39, 58, 84) and furthermore establish the loss of p16 and p21 functions as the minimum necessary requirements.

A recent report (4) presented evidence that human fibroblasts deficient solely in p16 function are resistant to Ras-induced senescence. The cells in that study were derived from a patient with a homozygous 19-bp deletion in the second exon of the *CDKN2A* locus that affects the coding region of both the

p16^{Ink4a} and p19^{ARF} proteins. Extensive evidence was presented by the researchers that p16 was completely inactive and that while Arf was expressed as a partially frameshifted protein, it retained full activity. Although this study at face value contradicts our finding of a requirement for the combined loss of p16 and p21, there are several intriguing parallels. Most importantly, in both cases, the introduction of Ras failed to arrest the cells, induced anchorage-independent growth with a small colony morphology in soft agar, and failed to produce tumors in nude mice. Furthermore, Brookes et al. (4) found no evidence for either activation of p53 or induction of p21 in response to Ras. This interesting finding, which is at odds with our results as well as those of several other groups (18, 30, 31, 58, 75, 84), nevertheless explains why the requirement to abolish p21 function was apparently absent in their experiments.
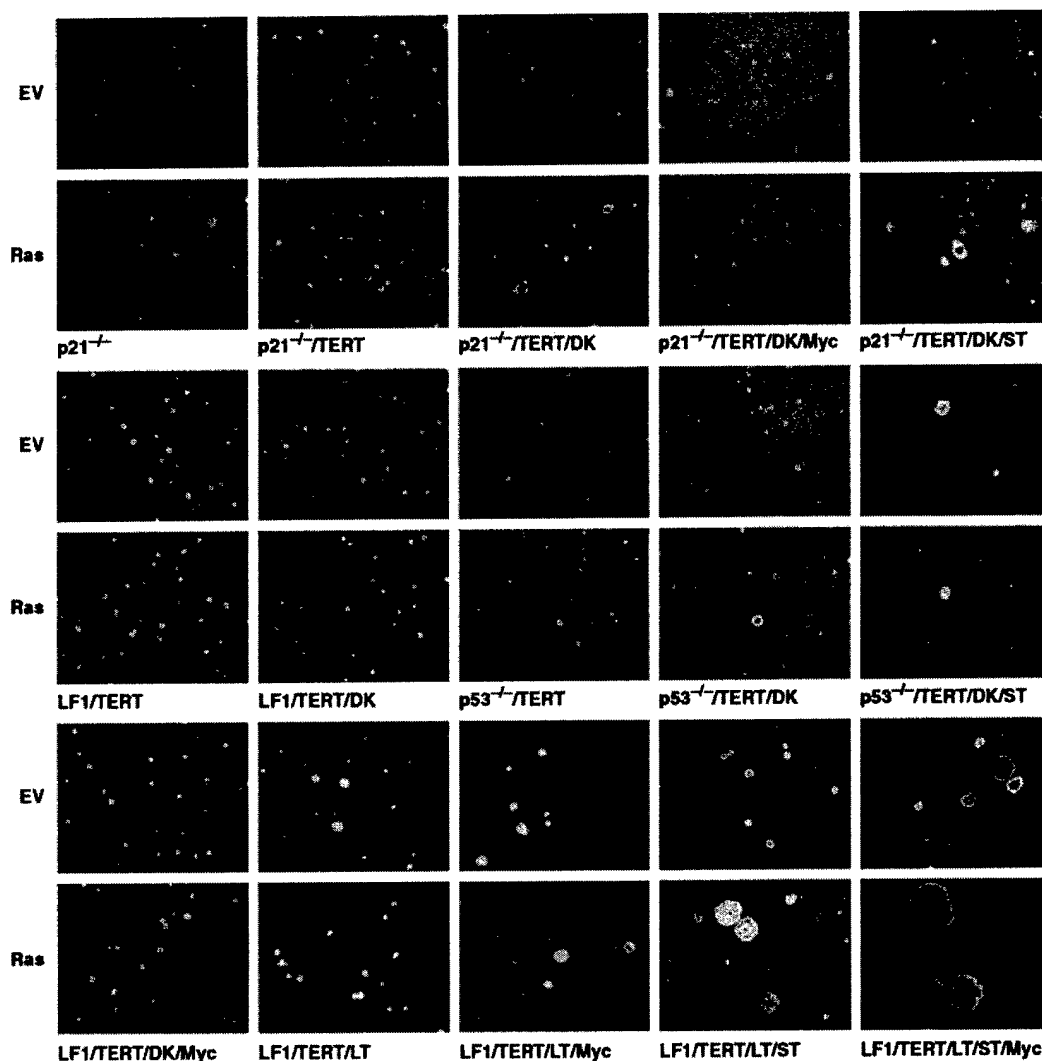
FIG. 5. Soft-agar colony assays. The indicated cell lines were infected with pBabe-mEYFP/Ras virus (Ras) or empty vector virus (EV) (pBabe-mEYFP) and propagated as described in the legend to Fig. 3. Six days after infection, cells were harvested by trypsinization, and 10^4 or 10^5 cells were plated in soft agar as indicated in Materials and Methods. Photomicrographs were taken under phase-contrast illumination 21 days after plating. All photomicrographs are shown on the same scale to illustrate relative colony sizes. It should be noted that $p21^{-/-}$TERT/DK/MYC cells appeared to disintegrate when placed in soft agar, rather than simply failed to proliferate.

The reasons why Ras can induce p21 in some experiments but not in other experiments are not clear and will require further study. Besides the usual differences in methodologies used by the different laboratories, the possibility that the frameshifted Arf protein expressed from the mutant *CDKN2A* locus possesses some degree of abnormal biological activity needs to be further evaluated. For example, Ras can affect p53 activity directly through Mdm2 (52), and the frameshifted Arf protein, through its ability to bind to Mdm2, may interact with this pathway.

Several lines of evidence indicate that the small soft-agar colonies produced by $p53^{-/-}$TERT/DK/Ras and $p21^{-/-}$ TERT/DK/Ras cells represent a biologically significant phenotype. First, colonies were observed in repeated trials and did not emerge from cells infected with empty vector or from cells in which either p16 or p21 functions were singly abolished. Second, when colonies were recovered from the soft agar and

expanded into cell lines, they retained ectopic Ras expression and replated in soft agar with similar plating efficiencies and colony morphology. Cells that did not express ectopic Ras could be recovered from the soft-agar plates but did not replate. These results indicate that the soft-agar colony formation requires Ras and is unlikely to depend on the acquirement of secondary mutations. The reasons for the slow growth in soft agar have not been explored in detail. One clear contributing factor is the slow growth of the parental $p53^{-/-}$TERT/DK and $p21^{-/-}$TERT/DK cells, which was not significantly accelerated by Ras. The fact that low oxygen conditions have been reported to promote soft-agar colony formation may explain, in part, why anchorage-independent growth of TERT-, LT-, and Ras-expressing cells was not previously documented. Thus, in addition to the study of Brookes et al. (4) discussed above, the data presented here are the first delineation of minimum genetic requirements in terms of defined cellular functions for

TABLE 2. Soft-agar plating efficiencies

Cell line	Plating efficiency[a]			
	Empty EYFP vector		EYFP-Ras vector	
	Total colonies[b]	Macro colonies[c]	Total colonies[b]	Macro colonies[c]
LF1/TERT	0	0	0	0
LF1/TERT/DK	0	0	0	0
LF1/TERT/DK/Myc	0	0	0	0
LF1/TERT/LT	54	0	53	0
LF1/TERT/LT/Myc	53	0	57	0
LF1/TERT/LT/ST	60	0	97	56
LF1/TERT/LT/ST/Myc	70	62	100	70
p21$^{-/-}$TERT	0	0	0	0
p21$^{-/-}$TERT/DK	0	0	18	0
p21$^{-/-}$TERT/DK/ST	0	0	20	0
p21$^{-/-}$TERT/DK/Myc	0	0	0	0
p53$^{-/-}$TERT	0	0	0	0
p53$^{-/-}$TERT/DK	0	0	21	0
p53$^{-/-}$TERT/DK/ST	12	0	24	0

[a] All plating efficiencies have been adjusted for the infection efficiency of each culture with the EYFP and EYFP-Ras vectors. Infection efficiencies were in the range of 20 to 40%. Plating efficiencies are expressed as relative percentages (see footnotes b and c). Absolute plating efficiencies (total colonies per cells plated, adjusted for infection efficiency) were also determined and were in the range of 1 to 5%. For example, the absolute plating efficiencies of LF1/TERT/LT/ST cells infected with EYFP-Ras, LF1/TERT/LT/ST/Myc cells infected with EYFP, and LF1/TERT/LT/ST/Myc cells infected with EYFP-Ras3 were 5.4, 5.9, and 5.7%, respectively.

[b] Plating efficiencies are expressed relative to the most efficient example, LF1/TERT/LT/ST/Myc cells infected with EYFP-Ras virus, which has been set at 100%. Total colonies are defined as all multicellular aggregates estimated to contain 30 to 100 cells and all macroscopic colonies. This category thus includes the small, normal, and large colony morphologies defined in the legend to Fig. 1 and depicted in Fig. 5.

[c] Plating efficiencies are expressed as a percentage of the total colonies for any given cell-virus combination. Macroscopic (Macro) colonies are defined as all colonies of >0.2 mm in diameter. This category thus includes the normal and large colony morphologies defined in the legend to Fig. 1 and depicted in Fig. 5. Note that macroscopic colonies were observed for only the LF1/TERT/LT/ST cells infected with EYFP-Ras virus, LF1/TERT/LT/ST/Myc cells infected with empty EYFP virus, and LF1/TERT/LT/ST/Myc cells infected with EYFP-Ras virus.

TABLE 3. Tumor formation[a]

Cell line	Animal	Time at sacrifice (wk)/tumor diameter (cm)
LF1/TERT/LT/ST	1	11/1.7
	2	17/1.5
	3	TF[b]
	4	TF
LF1/TERT/LT/ST/Myc	1	11/2.0, 7/2.0
	2	TF, 11/0.7
	3	TF, TF
	4	TF
BJELR	1	11/2.13
	2	11/1.70
	3	11/1.02
	4	TF

[a] Each cell line was injected into four animals, and each animal was injected with Ras-infected cells in the right flank and with control cells infected with empty virus in the left flank. The tumor data presented in the table are for Ras-infected cells; cells infected with empty vector did not form tumors in any of the animals. The following cell lines were also injected but did not display any tumor formation for up to 6 months: p21$^{-/-}$TERT, p21$^{-/-}$TERT/DK, p21$^{-/-}$TERT/DK/Myc, p21$^{-/-}$TERT/DK/ST, p53$^{-/-}$TERT/DK, p53$^{-/-}$TERT/DK/ST, LF1/TERT/DK, LF1/TERT/LT, LF1/TERT/DK/Myc, and LF1/TERT/LT/Myc. BJELR cells also exhibited tumors (two of three injected animals) in the NIH-bg-nu-xidBR mouse strain (Charles River) (data not shown). As controls, BJELT cells (18) were injected into the left flanks of animals receiving BJELR cells. BJELT cells did not form tumors in any of the animals. Two experiments were done with mice injected with the LF1/TERT/LT/ST/Myc cell line (three mice in the second experiment).

[b] TF, tumor-free.

anchorage-independent growth. We propose that anchorage-independent growth of a human fibroblast requires a minimum of three genetic events: activation of Ras and elimination of p16 and p21 functions. Whether activation of telomerase is required only for extension of life span or also has a component in the anchorage-independent phenotype itself (69, 81) is not addressed by our experiments, because the p21$^{-/-}$DK cell line was close to the end of its replicative life span at the time of Ras transformation. It also should be kept in mind that our results with fibroblasts may not apply to other cell types, for example epithelial cells, which may have very different requirements for immortalization and transformation.

Surprisingly, we observed that Ras-expressing p53$^{-/-}$TERT/DK/ST and p21$^{-/-}$TERT/DK/ST cells formed only small colonies in soft agar. Since we have previously shown that coexpression of a dominantly acting mutant of p53 (p53^{DD}), a Cdk4 mutant (Cdk4^{R24C}), and cyclin D1 functionally replace LT in the transformation of human fibroblasts (18), these observations suggest that the genetic abolition of p53 and the expression of the p53^{DD} mutant protein are not functionally equivalent. We make this suggestion because the coexpression of Cdk4^{R24C} and cyclin D1 should be equivalent to the expression of the DK fusion protein (49). However, the p53^{DD} mutant has

known gain-of-function properties, indicating that some of these other functions may participate in cell transformation (73). For example, the p53^{DD} protein not only binds wild-type p53, thus preventing the activation of p53-dependent transcriptional targets, but also interacts with p53 cellular partners such as Mdm2 and p300/CBP (59). The interaction of p53^{DD} with wild-type p53 also serves to stabilize p53 against turnover and acts as a substrate for many kinases that act on p53 (21). In addition, since p53 forms complexes with the related proteins, p63 and p73, the p53^{DD} mutant may perturb the functions of these proteins in addition to its effects on p53 (36, 72).

Moreover, LT has biological activities in addition to those that target cellular p53 and Rb proteins, and although the ability of LT to cooperate in the transformation of human cells does not appear to require its J domain (18), other effectors may also participate in transformation (66, 67, 71, 82, 83). For example, LT forms a trimeric complex with p53 and Mdm2 (1), and Mdm2 can promote growth arrest by p53-independent pathways (5, 9). Since Ras induces Mdm2 transcription through AP-1 and Ets sites in the Mdm2 promoter (52), the interaction of p53^{DD} with Mdm2 may antagonize Ras-induced, Mdm2-dependent, p53-independent growth inhibition. In addition, LT, the adenoviral E1A, and p53^{DD} proteins each interact with p53 and p300/CBP (1), and the interaction of E1A and p300/CBP was recently shown to play an important role in human fibroblasts transformed by E1A, Mdm2, and Ras (57). Since the genetic abolition of p53 severs this interaction, these observations suggest several plausible mechanisms that could explain why the p53$^{-/-}$TERT/DK/ST cells described in this report fail to form large, anchorage-independent colonies.

Moreover, since tumor-associated p53 mutants also exhibit gain-of-function properties (10), further work will be necessary to delineate the p53-related activities that conspire to transform human cells.

The ability of ST to promote tumor growth in conjunction with LT and Ras depends on its ability to bind PP2A (18, 43, 80). PP2A is a heterotrimeric serine/threonine phosphatase with numerous cellular targets and biological functions (23). Although PP2A is essential for viability (13, 15, 33), in a general sense its activity has antiproliferative effects (23). Its targets include (among many others) the mitogen-activated protein kinase pathway (63, 64), G_1 cyclin-dependent kinases (79), p70 S6 protein kinase (77), mitochondrial Bcl2 (54), and Mdm2 (42). ST binds and interferes with PP2A function (43, 80), which results in the upregulation of mitogen-activated protein kinase pathway activity and Ras signaling (64). The specific targets of PP2A that are relevant to the ability of ST to promote tumor growth have not been identified. However, it is unlikely that this function will be limited to a simple augmentation of Ras signaling, especially in the case of ST and Myc cooperation. This is evident from the observation that on the LF1/TERT/LT background, Myc and Ras cooperated only weakly, whereas Myc and ST showed strong cooperation. It should be noted that in the present study, the cooperative effects between Myc and ST were observed in the context of LT-expressing cells. Although it is reasonable that the function of LT in these experiments was to antagonize the pro-apoptotic effects of Myc and/or to alleviate the growth arrest caused by Ras, we were unable to test these possibilities more directly because of a lack of suitable drug-resistant retrovirus vectors for further genetic manipulations.

The major focus of this study was to continue to resolve a minimum human fibroblast transformation pathway into single and well-defined cellular functions. Previous work has defined four distinct categories of functional requirements: acquisition of indefinite life span, expression of growth- and transformation-promoting functions, elimination of growth-inhibitory effectors, and escape from apoptotic surveillance mechanisms (16, 19). The cellular p53 and Rb pathways are almost universally compromised in human cancers (62) and have also been strongly implicated in bypassing replicative senescence (3, 60). The extent to which mutations in these pathways are necessary for immortalization in addition to the expression of telomerase has been controversial (26, 47, 50, 61). The very significant need to compromise the p53 and Rb pathways is in large part due to their function in surveillance mechanisms. We have shown here that loss of p21 and p16 function is sufficient to escape the surveillance of inappropriate oncogenic signaling, and thus allow Ras to elicit limited anchorage-independent growth. p53 mutations are much more frequent in human cancers than p21 mutations; at least one reasonable explanation is that loss of p53 provides additional escape from many apoptotic surveillance mechanisms, which allows the expression of growth-promoting functions such as Myc. We have also shown that full transformation and tumorigenesis require ST and possibly another yet undefined function that can be contributed by LT. The precise definition of this activity and the targets of PP2A that are the likely downstream effectors of ST are currently under investigation.

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Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase

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Summary

Recent studies have demonstrated that introduction of *hTERT* in combination with SV40 large T antigen (LT), small t antigen (st), and H-*ras*V12 suffices to transform many primary human cells. In human mammary epithelial cells (HMECs) expressing elevated c-Myc, activated H-Ras is dispensable for anchorage-independent growth. Using this system, we show that st activates the PI3K pathway and that constitutive PI3K signaling substitutes for st in transformation. Moreover, using constitutively active versions of Akt1 and Rac1, we show that these downstream pathways of PI3K synergize to achieve anchorage-independent growth. At lower levels of c-*myc* expression, activated PI3K also replaces st to complement H-*ras*V12 and LT and confers both soft agar growth and tumorigenicity. However, elevated c-*myc* expression cannot replace H-*ras*V12 for tumorigenesis. These observations begin to define the pathways perturbed during the transformation of HMECs.

Introduction

Cultured HMECs immortalized with *hTERT* provide an important in vitro model system for studying the tumorigenic conversion of HMECs. HMECs must bypass several barriers before they become immortalized (Romanov et al., 2001). Soon after explantation, HMECs lose p16[INK4A] expression usually secondary to promoter methylation (Foster et al., 1998). The introduction of *hTERT* readily immortalizes such cells (Kiyono et al., 1998). We previously showed that the serial introduction of the SV40 early region and H-*ras*V12 sufficed to transform several types of *hTERT*-immortalized human cells, including early passage HMECs (Hahn et al., 1999; Elenbaas et al., 2001). We and others subsequently demonstrated that both LT and st antigens are required for the transforming activity of the SV40 early region (Yu et al., 2001; Hahn et al., 2002). Several groups have used other combinations of introduced genes to transform other human cells (Brookes et al., 2002; Seger et al., 2002). These observations suggest that dysregulation of a limited set of pathways governs the human cell transformation and that further dissection of the signaling pathways perturbed by these introduced genes will identify key steps in oncogenesis.

The *ras* gene is mutated in many human cancers. Ras pro-teins act as GTP/GDP-regulated molecular switches that modulate signal transduction pathways controlling cell proliferation, differentiation, and survival (Shields et al., 2000). Ras stimulates multiple effector-mediated signaling pathways, including the Raf-MEK-ERK, the phosphatidylinositol 3-kinase (PI3K), and the RalGDS pathways. Ras function in oncogenic transformation has been extensively studied; however, much of our understanding of Ras transformation is derived from rodent cell systems. The Raf effector pathway has been shown to be a key mediator of Ras-transforming activity in rodent cell lines (Cowley et al., 1994; Mansour et al., 1994). However, a recent report indicated that Ras-activated signaling via the RalGDS pathway plays a dominant role in the transformation of human cells in contrast to the central contribution of Raf signaling in rodent cells (Hamad et al., 2002). Thus, although alterations of the pathways regulated by Ras play an important role in transformation, the relative contribution of each of these signaling pathways remains undefined.

The SV40 early region encodes at least two proteins through alternative splicing. One of these oncoproteins, the SV40 LT antigen, binds to and inactivates, among other proteins, the p53 and retinoblastoma (pRB) tumor suppressors (Sullivan and Pipas, 2002). Inactivation of these two tumor suppressor path-

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S I G N I F I C A N C E

Studying the mechanisms used by DNA tumor viruses to transform mammalian cells has elucidated the identity and function of many cellular pathways critical for the development of human cancers. The SV40 early region encodes two oncoproteins LT and st that transform human cells. Although the actions of LT are well characterized, the pathways perturbed by st remain undefined. Here we show that one critical target of st is the PI3K pathway. Activation of PI3K signaling functionally mimics the expression of st and confers anchorage-independent growth and tumorigenicity. These human cells, which are dependent on activated alleles of PI3K or Akt for their transforming behavior, will facilitate the testing of specific inhibitors of this oncogenic pathway.

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ways suffices to transform human cells in the presence of onco-genic H-Ras, hTERT, and a second oncoprotein from the SV40 early region, st. Although the role of st in human cell transforma-tion was not appreciated in initial studies of *hTERT*-immortalized cells (Hahn et al., 1999; Elenbaas et al., 2001), st expression was subsequently shown to be a prerequisite for the oncogenic conversion of human cells (Yu et al., 2001; Hahn et al., 2002). Replacing the SV40 early region with human papillomavirus E6/E7 oncoproteins failed to transform human fibroblasts (Morales et al., 1999) and human keratinocytes (Yuan et al., 2002). The additional expression of st in these cells, expressing either LT or E6/E7, however, completed the transformation of such cells (Hahn et al., 2002; Yuan et al., 2002). Indeed, these observations confirm earlier studies using SV40 mutants unable to produce functional st, which showed that these mutants were defective in the transformation of human fibroblasts (de Ronde et al., 1989). This ability of st to cooperate in transformation depends upon its interaction with the PP2A family of serine-threonine phosphatases (Pallas et al., 1990; Yang et al., 1991; Mungre et al., 1994; Hahn et al., 2002). Although several signaling path-ways, including the MEK-ERK and PI3K/Akt pathways, are per-turbed by the interaction of st with PP2A (Sontag et al., 1993; Yuan et al., 2002), the identity of the particular pathways in-volved in human cell transformation remains obscure and over-laps with those perturbed by oncogenic Ras.

PI3Ks are heterodimers with a regulatory subunit, p85, and a catalytic subunit, p110. The primary consequence of PI3K activation is the conversion of phosphatidylinositol-4,5-diphos-phate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) in the plasma membrane, which then functions as a second messenger to activate downstream pathways that involve Akt, Rac1/Cdc42, and other proteins (Vanhaesebroeck et al., 2001). Numerous studies have highlighted the importance of PI3K function in cell proliferation and transformation. Amplification and aberrant activation of PI3K and Akt occur in breast and ovarian cancers (Bellacosa et al., 1995; Shayesteh et al., 1999). Moreover, PTEN, a negative regulator of the PI3K pathway, was originally isolated as a tumor suppressor gene in breast cancer and glioblastomas, and has subsequently been implicated in many types of human cancers (Li et al., 1997; Steck et al., 1999). Together, these observations underscore the importance of PI3K signaling in malignant transformation.

The c-Myc oncoprotein is also frequently amplified or over-expressed in naturally arising primary breast carcinomas (Escot et al., 1986). In fact, deregulated expression of c-*myc* occurs in a broad range of human cancers and is often associated with aggressive, poorly differentiated phenotypes (Pelengaris et al., 2002). The c-*myc* protooncogene regulates diverse biological processes, including cell proliferation, growth, and differentia-tion (Dang, 1999). Recent studies with conditional transgenic mouse models have shown that c-*myc* activation is required for solid tumor maintenance (Pelengaris et al., 1999), and c-*myc* inactivation results in tumor regression (Felsher and Bishop, 1999). However, the role of c-*myc* in tumorigenesis remains enigmatic.

Here we show that introduction of LT and st suffices to permit late passage HMEC-*hTERT* to grow in an anchorage-independent manner. Exploiting this system, we further demon-strate that activation of the PI3K pathway or activation of two downstream target pathways of PI3K, in the presence of in-creased expression of c-*myc*, replaces st and permits anchor-

age-independent growth. However, this combination fails to permit HMEC to form tumors in animal hosts without the addi-tional expression of H-*ras*V12. Since alterations in the pathways governed by PI3K, Ras, and Myc are associated with human breast cancer, these observations provide an opportunity to construct increasingly relevant models of human breast cancer that will not only increase our understanding of malignant trans-formation of breast epithelium but also provide new substrates for the discovery of novel therapeutics.

Results

Anchorage-independent growth of late passage HMEC-*hTERT* expressing SV40 LT and st

To dissect further the pathways involved in the transformation of HMECs, we obtained early and late passage HMECs. Consistent with prior observations (Foster et al., 1998; Romanov et al., 2001), these cells, which had bypassed an initial growth arrest (termed M0), lack expression of p16[INK4a] (data not shown). We immortalized early passage HMECs population doubling 20 (PD 20) through the introduction of *hTERT* and obtained commer-cially available, late passage *hTERT*-immortalized HMECs at PD 134, which maintain the phenotype and karyotype of normal epithelial cells.

To express SV40 T antigens in HMECs, we introduced either the SV40 early region, which encodes both LT and st, or LT and st individually by retrovirus-mediated gene transfer (Figure 1A). We also generated cell lines that express H-*ras*V12 in com-bination with these introduced genes and assessed the ability of each of these cell lines to proliferate in an anchorage-indepen-dent fashion, a hallmark of in vitro transformation (Cifone and Fidler, 1980). Surprisingly, expression of SV40 early region in the absence of H-*ras*V12 sufficed to induce colony formation in late passage HMECs-*hTERT* (Figure 1B). We found similar results when we introduced LT and st separately into late pas-sage *hTERT*-immortalized HMECs (Figure 1B). Consistent with previous observations (Elenbaas et al., 2001), early passage HMECs expressing *hTERT* and the SV40 early region required the addition of H-*ras*V12 for anchorage-independent growth. Moreover, expression of st alone in HMECs expressing *hTERT* failed to confer the ability to grow in an anchorage-independent manner (data not shown). Thus, although late passage *hTERT*-immortalized HMECs retain many morphological features of pri-mary HMECs, such cells require fewer alterations to permit anchorage-independent growth.

SV40 st enhances and prolongs EGF-induced Akt phosphorylation

Although the interaction of st with PP2A is essential for the transformation of human cells (Porras et al., 1996; Hahn et al., 2002), the signaling pathways regulated by this interaction and necessary for human cell transformation remain undefined. In addition, the presence of H-*ras*V12 adds considerable complex-ity by activating multiple signaling pathways, many of which are also PP2A targets. Thus, these late passage HMECs-*hTERT* that do not require constitutively active Ras for anchorage-independent growth provided a system with which to study the effects of st without the confounding effects of oncogenic Ras.

Transient expression of st in mammalian cells activates growth factor-stimulated signaling pathways involving the PI3K (Sontag et al., 1997; Garcia et al., 2000) and MEK-ERK pathways
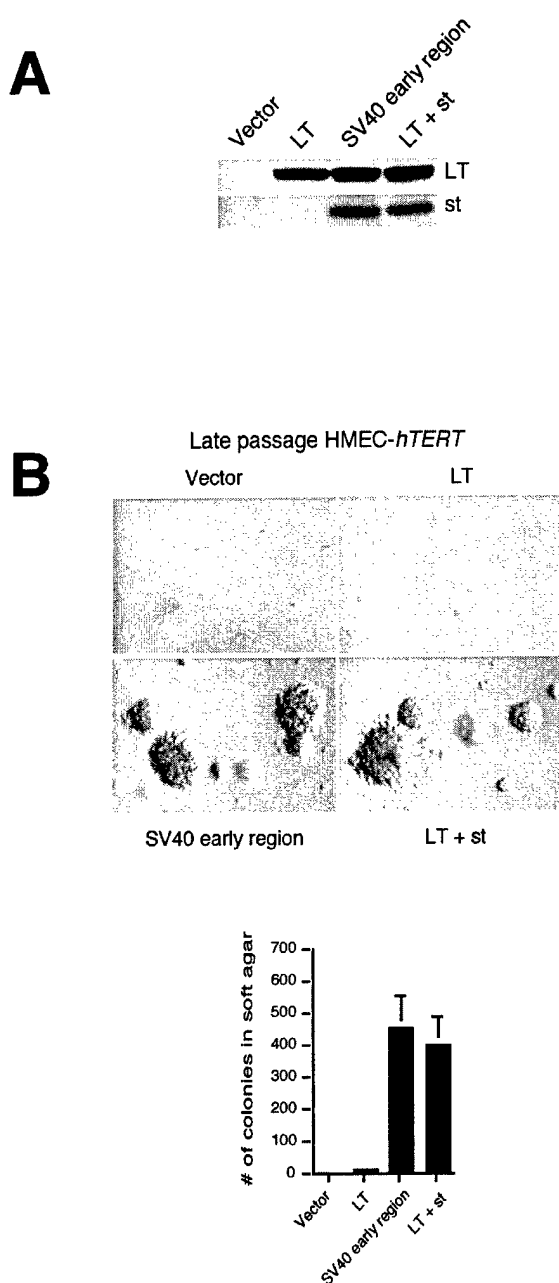
A

B

Late passage HMEC-*hTERT*

Vector | LT

SV40 early region | LT + st

Figure 1. Anchorage-independent growth of late passage HMECs-*hTERT* expressing LT and st

A: Immunoblot analysis of cells expressing LT and/or st. Sixty micrograms of soluble cell lysates were separated on 10% gels and blotted with a monoclonal antibody (mAb) specific for the SV40 early region.

B: Soft agar growth of late passage HMEC-*hTERT* expressing SV40 early region, or cDNAs encoding LT and st, respectively, as indicated. 5×10^4 cells were seeded per 60 mm plate, and the number of soft agar colonies (≥ 0.2 mm in diameter) was scored after 3 weeks. The means $\pm$ SD for three experiments are shown.

(Sontag et al., 1993; Ugi et al., 2002). To assess the effects of st in HMECs on these two signaling pathways, we measured the level of phosphorylation of Akt and ERK in HMECs-*hTERT*-LT stably expressing st. Cells were starved in mammary epithelial basal medium (MEBM) and stimulated with epidermal growth factor (EGF). Cell lysates were analyzed by immunoblotting with antibodies specific for activated Akt (phosphorylated at Ser473) or activated ERK1/2 (phosphorylated at Thr202/Tyr204). The expression of st did not alter basal levels of phospho-Akt or phospho-ERK1/2 (Figures 2A and 2B). However, upon stimulation with EGF, phospho-Akt levels were further enhanced by 2- to 3-fold in st-expressing cells (Figure 2A), whereas the phospho-ERK levels were slightly reduced (Figure 2B). We observed similar results when we interrogated the phosphorylation state of Akt at Thr308 (data not shown). We also examined alterations in Akt phosphorylation upon EGF stimulation in HMECs over time. Significantly, following EGF stimulation, we observed sustained levels of phosphorylated Akt in st-expressing cells (Figure 2C), while in control cells, Akt phosphorylation was maximal at 10 min and declined to basal levels 60 min after EGF stimulation (Figure 2C). These effects of st on Akt phosphorylation in HMECs did not depend on the presence of LT (data not shown). Thus, expression of st permits enhanced and prolonged phosphorylation of Akt in HMECs.

st-induced HMEC transformation requires PI3K function

To investigate whether st-induced HMEC transformation requires PI3K signaling, we used a well-characterized dominant-negative mutant of the PI3K regulatory subunit, p85, lacking the binding site for the catalytic subunit, p110 (Dhand et al., 1994). This mutant, designated Δp85, strongly inhibits PI3K-dependent Ras-induced transformation of NIH3T3 cells (Rodriguez-Viciana et al., 1997). We introduced this Δp85 mutant or a control vector into late passage HMECs-*hTERT* expressing LT and st. The expression level of Δp85 in these cells was equivalent to that of endogenous p85 (Figure 3A), and the cells proliferated normally under standard conditions (data not shown). However, when we tested these cells for their ability to grow in an anchorage-independent fashion, we found that introduction of Δp85 into HMECs-*hTERT*-LT-st abolished colony formation (Figure 3B), indicating that the PI3K pathway participates in the st-mediated transformation of human cells.

Activation of PI3K permits anchorage-independent growth of late passage HMECs-*hTERT*-LT

To test the direct involvement of the PI3K pathway in the transformation of HMECs, we introduced an activated allele of PI3K, Myr-FLAG-p110α, into late passage HMECs-*hTERT* expressing LT. This FLAG epitope-tagged Myr-p110α is membrane-targeted and constitutively activated by N-terminal myristoylation (Klippel et al., 1996). The ectopically expressed Myr-FLAG-p110α protein in HMECs was verified by immunoprecipitation with anti-FLAG M2-agarose and immunoblotting with anti-FLAG antibody (Figure 4A). Endogenous Akt proteins were phosphorylated with or without EGF stimulation in HMECs expressing Myr-FLAG-p110α (Figure 4B), confirming the constitutive activation of Myr-FLAG-p110α. Expression of Myr-FLAG-p110α in HMECs-*hTERT*-LT resulted in growth of colonies in soft agar (Figure 4C) similar to that induced by st (Figure 1B), indicating
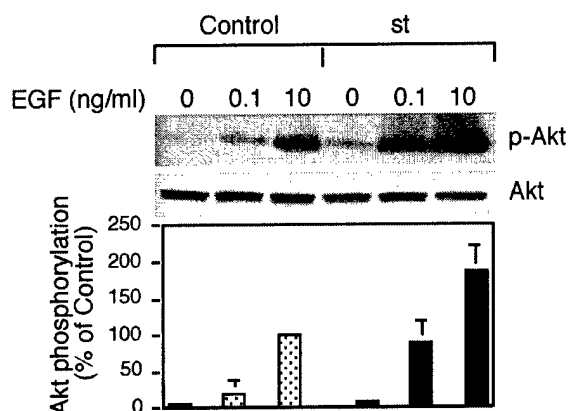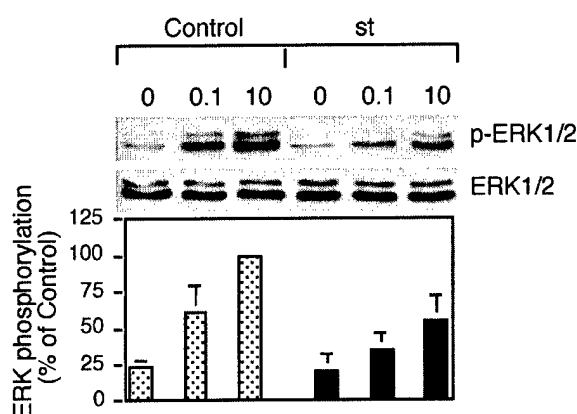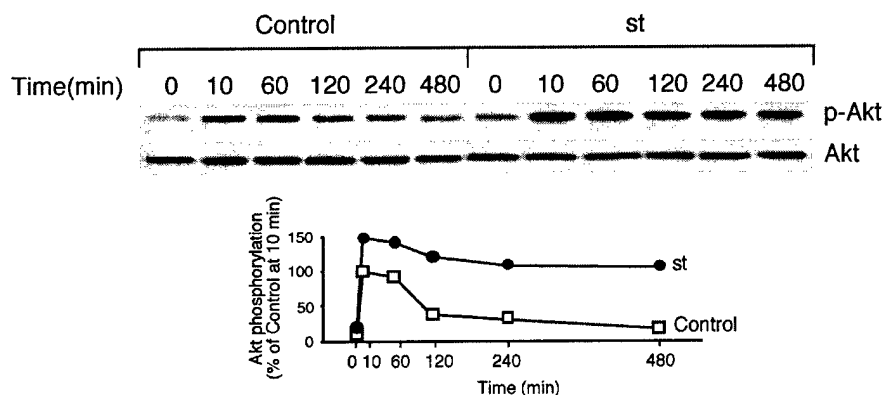
A

B

C

Figure 2. Effect of st on phosphorylation of Akt and ERK

A: Expression of st increases Akt phosphorylation. Cells were starved in MEBM overnight and then stimulated with EGF (0.1 ng/ml or 10 ng/ml) for 5 min. Cell lysates were analyzed by immunoblotting with antibodies specific to phospho-Akt (Ser473) or total Akt. Data are presented as the percentage of Akt phosphorylation when compared with control cells stimulated with EGF (10 ng/ml) and are the means ± SD for three experiments (lower panel).
B: Expression of st decreases the ERK phosphorylation. Cells were treated as in (**A**), and cell lysates were analyzed by immunoblotting with antibodies specific to phospho-ERK1/2 or total ERK1/2. Data are presented as the percentage of ERK phosphorylation when compared with control cells stimulated with EGF (10 ng/ml). The means ± SD for three experiments are shown (lower panel).
C: Expression of st prolongs EGF-induced phosphorylation of Akt. Cells were starved in MEBM overnight and stimulated with EGF (10 ng/ml) for the indicated time periods. Cell lysates were immunoblotted with antibodies specific to phospho-Akt (Ser473) or total Akt. Data are presented as the percentage of Akt phosphorylation when compared with control cells stimulated with EGF for 10 min (lower panel). The means ± SD for three experiments are shown.
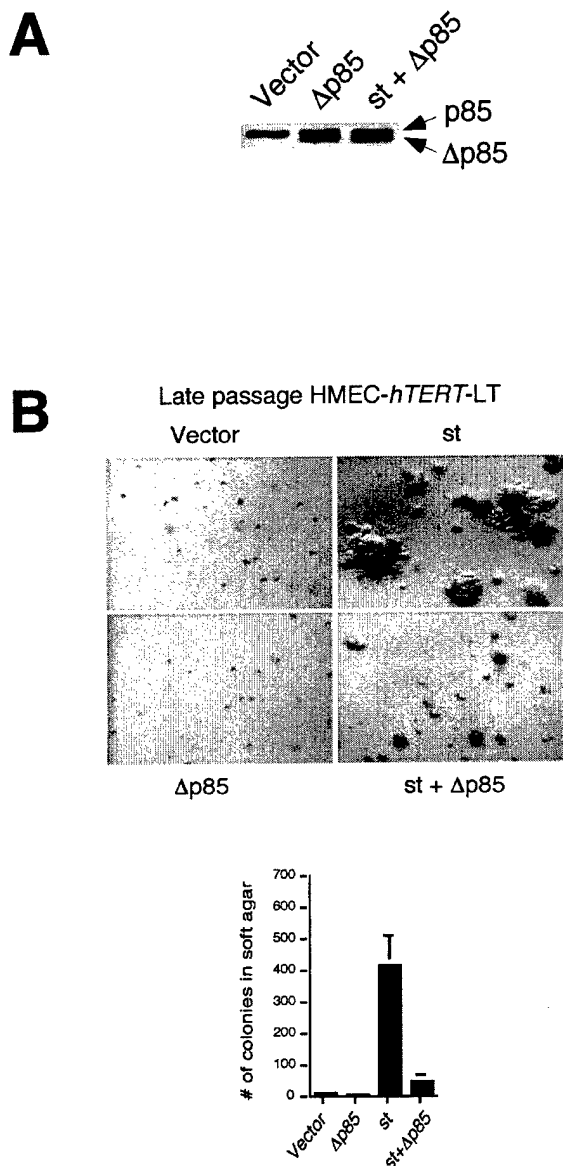
A

B

Late passage HMEC-*hTERT*-LT

A

B

C

Late passage HMEC-*hTERT*-LT

Figure 3. Δp85 inhibits the soft agar colony formation of late passage HMECs-*hTERT*-LT expressing st

A: Immunoblot analysis of cells expressing Δp85 in the presence or absence of st with a mAb specific for p85.

B: Soft agar growth of HMECs-*hTERT*-LT expressing st is inhibited by Δp85. The assay was carried out as described in Experimental Procedures, and the means ± SD for three experiments are shown.

that activation of PI3K substitutes for the expression of st to transform the late passage HMEC-*hTERT*-LT.

Coactivation of Akt1 and Rac1 permits anchorage-independent growth of late passage HMECs-*hTERT*-LT

PI3K signaling leads to the activation of several distinct signaling pathways. To determine which PI3K-dependent pathways par-
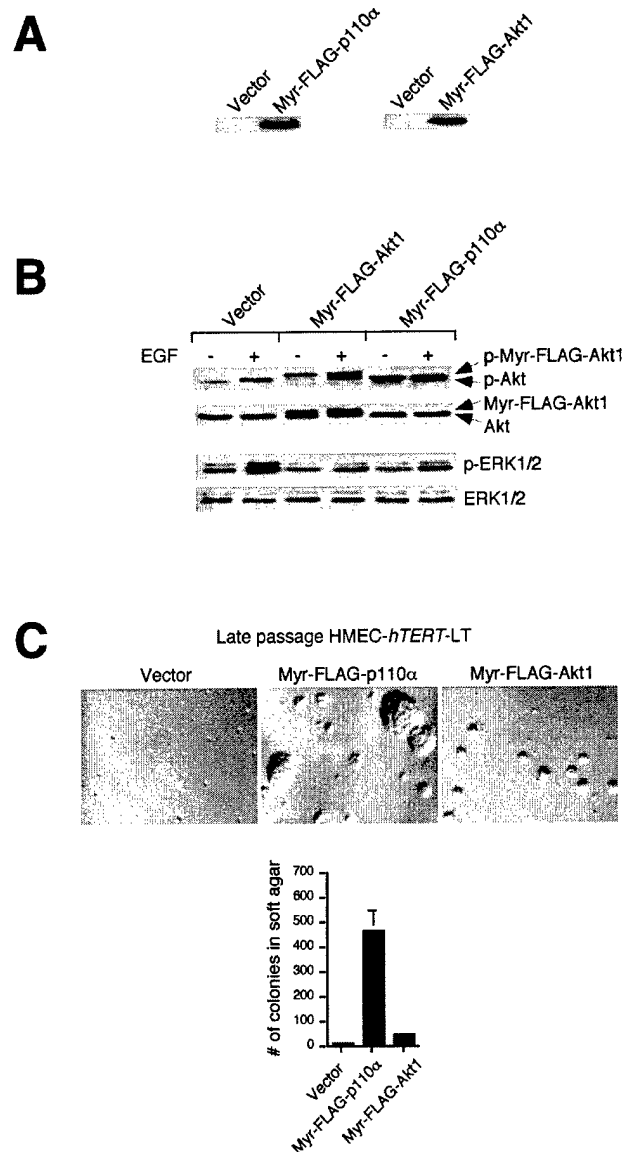
Figure 4. Anchorage-independent growth of late passage HMECs-*hTERT*-LT expressing Myr-FLAG-p110α or Myr-FLAG-Akt1

A: Expression of Myr-FLAG-p110α or Myr-FLAG-Akt1 was confirmed by immunoprecipitation with anti-FLAG M2 affinity agarose gel and immunoblotting with an anti-FLAG antibody.

B: Phosphorylation of Akt and ERK1/2 in cells expressing Myr-FLAG-p110α or Myr-FLAG-Akt1 in the presence or absence of EGF stimulation. Cells were starved in MEBM overnight and treated with EGF (10 ng/ml) for 5 min. Cell lysates were analyzed by immunoblotting with antibodies specific for phospho-Akt (Ser473), Akt, phospho-ERK1/2, or ERK1/2. Both forms of Myr-FLAG-Akt1 and endogenous Akt are revealed by immunoblotting with an anti-Akt antibody. Akt is phosphorylated in cells expressing Myr-FLAG-Akt1or Myr-FLAG-p110α independent of growth factor stimulation. Expression of Myr-FLAG-Akt1or Myr-FLAG-p110α reduced the phosphorylation level of ERK1/2.

C: Soft agar growth of late passage HMECs-*hTERT*-LT expressing Myr-FLAG-p110α. Many small colonies (~0.1 mm in diameter) were developed in cells expressing Myr-FLAG-Akt1 after 3 weeks. The means ± SD for three experiments are shown.

ticipate in HMEC transformation, we studied the transforming activities of several downstream targets of PI3K. Since st expression led to increased and prolonged phosphorylation of Akt, we introduced a membrane-targeted, epitope-tagged, constitutively active allele of Akt1 (Myr-FLAG-Akt1) into late passage HMEC-*hTERT*-LT cells (Figure 4A). This myristoylated Akt1 is constitutively phosphorylated regardless of the presence of growth factors such as EGF, whereas the endogenous Akt is only phosphorylated in the presence of EGF (Figure 4B). In HMECs expressing Myr-FLAG-Akt1 or Myr-FLAG-p110α, we observed downregulation of ERK1/2 phosphorylation levels (Figure 4B), similar to the levels seen in HMECs-*hTERT* expressing st (Figure 2B). This observation is consistent with previous observations that the Raf-MEK-ERK pathway is inhibited by activation of the PI3K/Akt pathway in C2C12 myoblasts, HEK293 cells, and MCF7 breast cancer cells (Rommel et al., 1999; Zimmermann and Moelling, 1999). While these cells formed small colonies in soft agar (Figure 4C), no large colonies appeared, even after extended incubation, indicating that while the myristoylated Akt exhibits constitutive activity, it is unable to allow these HMEC cells to grow in soft agar. These observations suggest that other downstream targets of PI3K, besides Akt, are required in concert to fully transform these HMECs.

Rac1, a member of the Rho family of GTPases, functions downstream of PI3K (Hawkins et al., 1995; Reif et al., 1996; Welch et al., 1998). Like Akt, Rac1 can be rendered oncogenic (Rodriguez-Viciana et al., 1997; Urich et al., 1997). We tested whether Rac1 is activated in HMECs-*hTERT*-LT stably expressing active PI3K. Cells were starved of growth factors, and Rac-GTP, the active form of Rac, was precipitated from fresh lysates using human PAK-1 PBD (p21 binding domain) agarose, which specifically binds the GTP bound form of Rac (Benard et al., 1999). Using an antibody specific for active Rac1, we observed elevated Rac-GTP levels in HMECs expressing Myr-FLAG-p110α or expressing H-rasV12 (Figure 5A), which is known to activate Rac (Nimnual et al., 1998; Scita et al., 1999), suggesting that Rac is regulated by PI3K in HMECs. Rac-GTP levels were not elevated in HMECs-*hTERT*-LT cells expressing st without growth factor stimulation (Figure 5A). Similar to the effect of st on Akt activation (Figure 2C), Rac-GTP levels were sustained in HMECs-*hTERT*-LT-st following EGF stimulation (Figure 5B), indicating that st modulates Rac as well as Akt.

We then investigated whether coexpression of activated Akt1 and Rac1 was sufficient for transformation. We cointroduced constitutively active Rac (Rac1V12) and Myr-FLAG-Akt1, singly and in combination, into late passage HMEC-*hTERT*-LT and verified the expression and activation of Rac1V12 in HMECs (Figure 5A). Unfortunately, we were unable to obtain HMECs-*hTERT*-LT expressing Rac1V12 alone, perhaps in part because Rho GTPases, including Rac1 and Cdc42, provoke proapoptotic pathways through JNK signaling (Kimmelman et al., 2000), which may be offset by the activation of the PI3K/Akt pathway (Murga et al., 2002). We found that only the coexpression of Rac1V12 and Myr-FLAG-Akt1 in late passage HMEC-*hTERT*-LT permitted anchorage-independent growth (Figure 5C). While these observations suggest that coactivation of Akt and Rac signaling may substitute for activation of PI3K to permit in vitro HMEC transformation, they do not eliminate the critical participation of other pathways downstream of PI3K.

Effects of PI3K signaling on cell proliferation

In order to assess the effects of st and PI3K pathway signaling on cell proliferation, we characterized the growth properties of HMEC-*hTERT*-LT cells stably expressing st, Myr-Flag-p110α, Myr-Flag-Akt1, or Myr-Flag-Akt1 and Rac1V12. Proliferation was determined by assessing relative cell accumulation at various times post-plating (Serrano et al., 1997). In fully supplemented mammary epithelial growth medium (MEGM), each of these cells displayed growth rates similar to those of cells expressing the control vector (Figure 6A). However, when the cells were maintained in culture medium supplemented with 0.5% of the normal growth factors, only cells expressing st, Myr-Flag-p110α, or Myr-Flag-Akt1 and Rac1V12 were able to grow (Figure 6B). These cells were the same cells previously found to be capable of anchorage-independent growth. Cells expressing control vector or Myr-Flag-Akt1 alone were unable to proliferate under starved conditions (Figure 6B). These observations show that transformed HMECs have reduced requirements for extracellular growth-promoting factors.

c-Myc and PI3K suffice to transform early passage HMECs and BJ human fibroblasts in vitro in the presence of *hTERT* and LT

The observation that st or activation of PI3K signaling was sufficient to confer anchorage-independent growth to late passage HMEC-*hTERT*-LT in the absence of oncogenic Ras suggested that, with passage, these *hTERT*-immortalized HMECs acquired genetic alterations that contribute to transformation. Wang et al. had previously shown that elevated levels of c-Myc occur in *hTERT*-immortalized HMECs cultured for more than PD 107 despite retaining a normal karyotype (Wang et al., 2000). Indeed, we confirmed that the late passage HMEC-*hTERT* cells used in these studies express higher levels of c-Myc protein than those of early passage cells (Figure 7A).

To determine the role of c-Myc oncoprotein in human cell transformation, we stably introduced c-*myc* into early passage HMEC-*hTERT* cells (PD 20) (Figure 7A) and subsequently introduced LT and st, Myr-Flag-p110α, or Myr-Flag-Akt1 and Rac1V12. The serial introduction of these genes required PD 30. These cells and all control cells were tested at PD 50 for their ability to grow in an anchorage-independent fashion. Expression of st, Myr-Flag-p110α, or Myr-Flag-Akt1 and Rac1V12 alone failed to transform early passage HMECs-*hTERT*-LT. However, the additional expression of c-*myc* or H-rasV12 into these early passage HMECs conferred the ability to grow in an anchorage-independent manner (Figure 7B and data not shown), indicating that the elevated expression of c-*myc* functionally replaces the expression of active H-ras to promote anchorage-independent growth of HMECs.

We further tested whether activation of PI3K and overexpression of c-*myc* also conferred anchorage-independent growth upon another human cell type. We stably introduced c-*myc* into early passage *hTERT*-immortalized human foreskin fibroblasts (BJ) (Hahn et al., 1999) (Figure 7A), and subsequently introduced LT and st or Myr-Flag-p110α. Similar to our observations in HMECs, we found that the combination of c-*myc* and st or Myr-Flag-p110α in BJ-*hTERT*-LT permitted colony formation in soft agar (Figure 7C), suggesting that functions of PI3K and c-*myc* in transformation are not unique to HMECs. Indeed, the combination of c-*myc*, LT, st, and *hTERT* also suffice to transform another strain of human fibroblasts, LF-1 (Wei et al.,
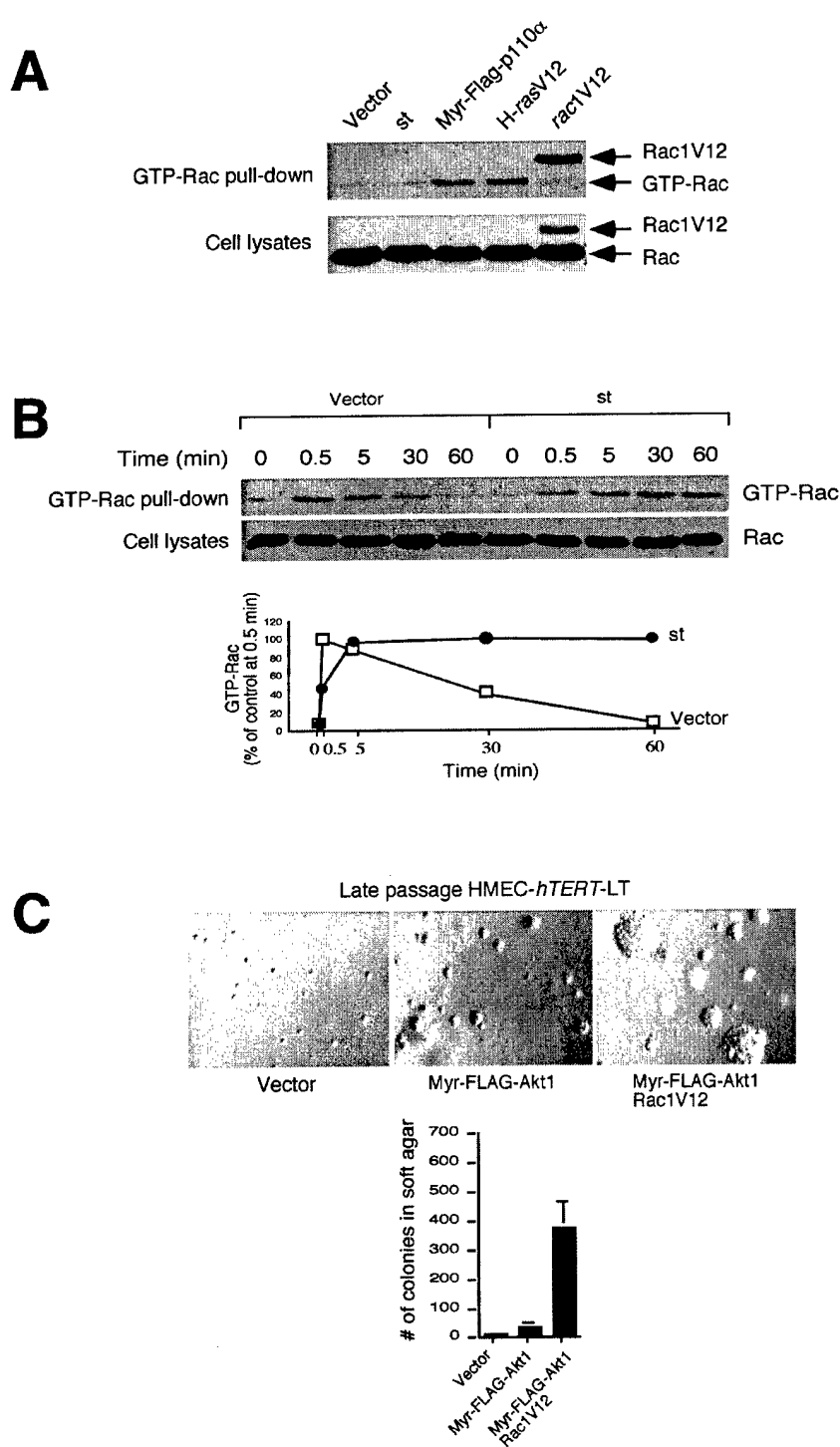
A

GTP-Rac pull-down

Cell lysates

Vector st Myr-Flag-p110α H-rasV12 rac1V12

← Rac1V12
← GTP-Rac

← Rac1V12
← Rac

B

Vector ──────── st

Time (min) 0 0.5 5 30 60 0 0.5 5 30 60

GTP-Rac pull-down ──────────────────── GTP-Rac

Cell lysates ──────────────────── Rac

GTP-Rac (% of control at 0.5 min)

120 100 80 60 40 20 0

0 0.5 5 ... 30 ... 60
Time (min)

st
Vector

C

Late passage HMEC-*hTERT*-LT

Vector   Myr-FLAG-Akt1   Myr-FLAG-Akt1 Rac1V12

# of colonies in soft agar

700 600 500 400 300 200 100 0

Vector Myr-FLAG-Akt1 Myr-FLAG-Akt1 Rac1V12

Figure 5. Anchorage-independent growth of late passage HMECs-*hTERT*-LT coexpressing Myr-FLAG-Akt1 and Rac1V12

A: Rac1V12 expression and Rac activation in HMECs. Cells were starved in MEBM overnight, and fresh cell lysates were prepared for GTP-Rac affinity precipitation. Immunoblot assays with anti-Rac were performed on pulldowns and cell lysates. Rac1V12 is highly activated in the absence of any growth factor. The activation of endogenous Rac is elevated in HMECs expressing Myr-FLAG-p110α and H-rasV12 without growth factor stimulation.

B: Rac activation is sustained in HMECs expressing st upon EGF stimulation. Cells were starved in MEBM overnight and treated with EGF (10 ng/ml) for the time indicated. Fresh cell lysates were prepared for GTP-Rac affinity precipitation. Immunoblot assays with anti-Rac were performed on both pulldowns and cell lysates. Data are presented as the percentage of Rac activation when compared with control cells stimulated with EGF for 0.5 min (lower panel).

C: Soft agar growth of late passage HMECs-*hTERT*-LT coexpressing Myr-FLAG-Akt1 and Rac1V12. The means ± SD for three experiments are shown.

A

(graph: Relative cell number vs Time (days), legend: Vector, st, Myr-p110a, Myr-Akt1, Myr-Akt+Rac1V12)

B

(graph: Relative cell number vs Time (days), legend: Vector, st, Myr-p110a, Myr-Akt1, Myr-Akt+Rac1V12)
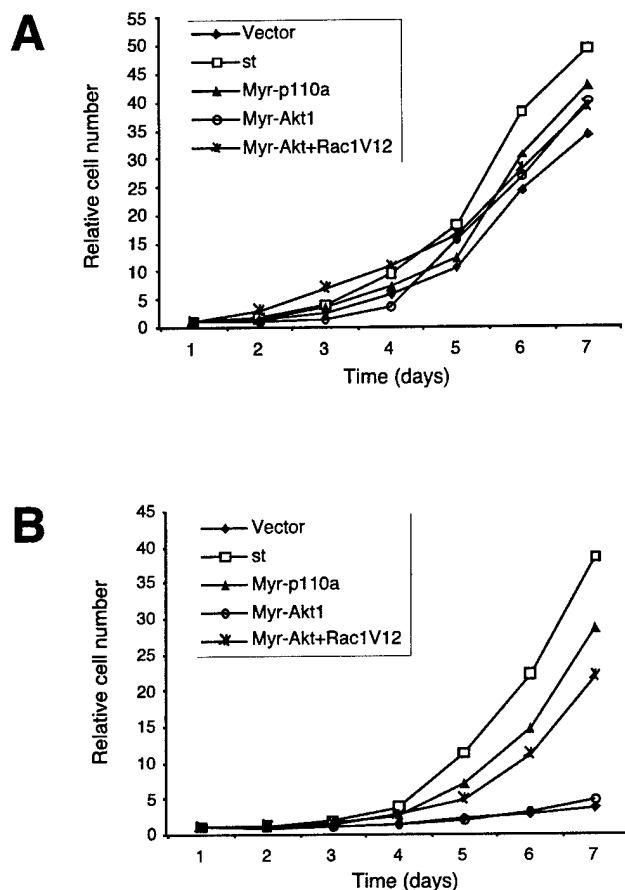
Figure 6. Growth properties of HMECs-hTERT-LT expressing st, Myr-FLAG-p110α, Myr-FLAG-Akt1, or Myr-FLAG-Akt1 and Rac1V12

A: Cells were grown in medium supplemented with growth factors at the concentrations suggested by the manufacturer.
B: Cells were grown in medium supplemented with a reduced (0.5%) concentration of the growth factors used in (**A**).

2003). Together, we conclude that in the setting of the expression of hTERT and LT, PI3K and c-myc functionally replace st and H-rasV12 to enable human cells to grow in an anchorage-independent manner.

Activated PI3K replaces st for tumor growth in HMECs but overexpression of c-myc fails to replace the requirement for H-rasV12

Although anchorage-independent growth often correlates with tumorigenicity, we determined whether the introduction of PI3K and c-myc into HMECs conferred the ability to form tumors in animal hosts. Since mixing of human mammary fibroblasts (HMFs) or Matrigel provides a more physiologic microenvironment that abrogates the long latency and inefficient growth of HMEC-derived tumors (Noel et al., 1993; Elenbaas et al., 2001), we introduced HMECs expressing various combinations of oncogenes mixed with normal HMFs or Matrigel into immunodeficient mice. Expression of st or Myr-Flag-p110α was sufficient to enable late passage HMECs-hTERT-LT bearing elevated c-myc

expression to grow in soft agar, but failed to promote tumor formation in vivo (Table 1).

Since high-level expression of H-rasV12 was required for tumor formation of HMECs expressing hTERT, LT, and st (Elenbaas et al., 2001), we tested whether a critical threshold level of c-myc expression is also required for tumorigenic transformation of HMECs. Ectopically introducing c-myc into late passage HMECs-hTERT-LT expressing st or Myr-Flag-p110α in addition to the already elevated endogenous expression level of c-myc also failed to permit these cells to produce tumors in animal hosts (Table 1). The additional introduction of H-rasV12 into these cells expressing hTERT, LT, and st or Myr-Flag-p110α promoted efficient tumor formation (Table 1). Consistent with these findings, we noted that the cointroduction of Myr-Flag-p110α and H-rasV12 induced higher levels of Akt phosphorylation than were observed in cells expressing either one of these elements singly (Figure 7D). Moreover, since HMECs expressing hTERT, LT, and H-rasV12, but lacking st or Myr-Flag-p110α, were unable to develop tumors (Table 1), these observations indicate that, although Ras activates PI3K signaling, the introduction of an activated allele of PI3K conferred additional signals required to convert human cells into tumorigenic cells.

Discussion

Here we demonstrate that activation of the PI3K pathway plays a crucial role in HMEC transformation. Since targets of st and H-Ras overlap in significant ways, the observation that late passage HMECs-hTERT expressing LT and st in the absence of H-rasV12 are capable of anchorage-independent growth greatly facilitated our study of the effects of st in cell transformation by eliminating the confounding effects of oncogenic Ras. One critical activity of st in cellular transformation is its ability to interact with and inhibit PP2A, a family of serine/threonine phosphatases (Mungre et al., 1994; Porras et al., 1996). As a consequence of PP2A inhibition by st, the phosphorylation states of many cellular kinases are altered (Janssens and Goris, 2001). Our observations indicate that the PI3K pathway plays an essential role in the transformation of HMECs and that st perturbs the physiological regulation of PI3K activity to facilitate cell transformation.

Although it is clear that SV40 st perturbs PI3K signaling, this viral oncoprotein may target the PI3K pathway at several levels. Expression of st does not alter tyrosine phosphorylation of the insulin, IGF-1, or EGF receptors (Ugi et al., 2002). A direct interaction between st and PI3K has not yet been demonstrated. Expression of st failed to increase Akt phosphorylation in the absence of EGF stimulation, but instead phosphorylation of Akt is increased and sustained upon EGF stimulation in st-expressing cells. These findings are consistent with a model in which st functions at the level of Akt (Yuan et al., 2002). In contrast, the mechanism by which st affects Rac activity remains unknown. The results presented here are reminiscent of a recent study that showed that expression of st in MDCK cells led to elevated activity and expression of Rac1 and Cdc42 and disorganization of the actin cytoskeleton (Nunbhakdi-Craig et al., 2003). Increasing evidence indicates that serine/threonine phosphorylation of Rho/Rac guanine nucleotide exchange factors (GEFs), such as Vav, Tiam-1, and PIX, are important for activation of Rac (Fleming et al., 1997; Bustelo, 2000; Shin et al., 2002).
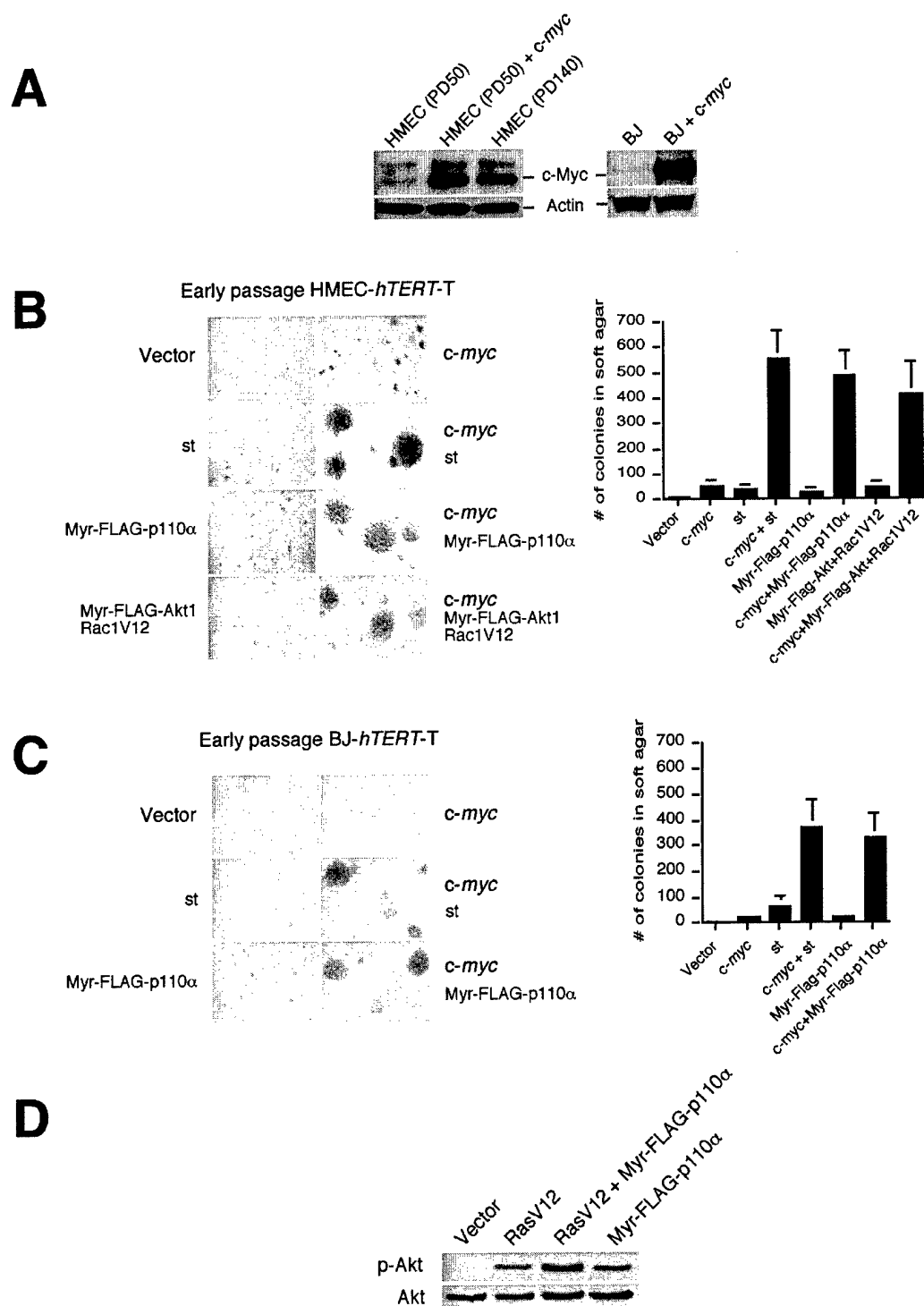
Figure 7. c-myc and Myr-FLAG-p110α cooperate to permit anchorage-independent growth of early passage HMECs and BJ fibroblasts expressing hTERT and LT

A: The expression of c-Myc protein. Cell lysates were prepared from early passage HMECs-hTERT (PD 50) and BJ fibroblasts in the absence or presence of ectopic c-myc expression and late passage cells (PD 140). The protein levels of c-Myc and actin from cells indicated were analyzed by immunoblotting.
B: Soft agar growth of early passage HMECs-hTERT-LT expressing st, Myr-FLAG-p110α, or Myr-FLAG-Akt1 and Rac1V12 in the presence of c-myc. The means ± SD for three experiments are shown.
C: Soft agar growth of early passage BJ-hTERT-LT cells expressing st or Myr-FLAG-p110α in the presence of c-myc.
D: Phosphorylation of Akt in HMEC-hTERT-LT cells expressing Myr-FLAG-p110α and RasV12. Cells were starved in MEBM overnight. Cell lysates were analyzed by immunoblotting with antibodies specific for phospho-Akt (Ser473).

Table 1. Formation of subcutaneous tumors in nude mice

Cells	Number of tumors/injection	
	with HMF	with Matrigel
HMEC/hTERT	0/3	
HMEC/hTERT, T	0/3	
HMEC/hTERT, T, t	0/6	0/3
HMEC/hTERT, T, Myr-Flag-p110α	0/6	0/3
HMEC/hTERT, T, t, c-Myc	0/6	0/3
HMEC/hTERT, T, Myr-Flag-p110α, c-Myc	0/6	0/3
HMEC/hTERT, T, t, RasV12	9/9	6/6
HMEC/hTERT, T, Myr-Flag-p110α, RasV12	6/6	3/3
HMEC/hTERT, T, RasV12	0/6	0/3
HMF	0/3	

For each injection, 2×10^6 cells of the indicated populations mixed with 2×10^6 cells of HMF, or 4×10^6 cells of HMECs mixed with Matrigel, were injected subcutaneously in a volume of 200 μl. Mice were sacrificed when tumors reached a diameter of 1 to 1.2 cm or after 4 months of monitoring.

Our studies indicate that activation of Akt is not necessarily synonymous with PI3K activation. Akt, a major target of PI3K, was identified as a key regulator of cell survival and proliferation and has been implicated in oncogenesis (Datta et al., 1999; Vivanco and Sawyers, 2002). Although Akt1 activity is often elevated in breast and prostate cancers (Sun et al., 2001), a constitutively active allele of Akt1 failed to replace active PI3K to promote the transformation of HMECs. However, the additional expression of Rac1V12 with Myr-FLAG-Akt1 achieved efficient colony formation in soft agar. Although we have not exhaustively tested all PI3K targets for their ability to complement Akt, the pathway regulated by Rac1 is an attractive candidate since Rac1 is a protooncogene implicated in human cancers (Sahai and Marshall, 2002).

The direct cooperation of PI3K and c-Myc in human cell transformation suggests that a functional connection between these oncogenic pathways exists. Several studies have shown that antiapoptotic proteins, such as Bcl2 and Bmi1, cooperate strongly with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis (Reed et al., 1990; Fanidi et al., 1992; Jacobs et al., 1999). This relationship between an antiapoptotic factor and c-Myc also applies to PI3K/Akt and c-Myc since c-Myc-induced apoptosis can be suppressed by activation of PI3K/Akt (Kauffmann-Zeh et al., 1997). Recent studies have shown that coordination of c-Myc and PI3K is responsible for cell cycle progression in fibroblasts (Jones and Kazlauskas, 2001). Similarly, in *Drosophila*, an activated form of Ras has been shown to promote cell growth and G1/S progression by increasing Myc and activating PI3K signaling (Prober and Edgar, 2002).

Is the observation that elevated c-Myc expression can functionally substitute for oncogenic H-Ras in the anchorage-independent growth of *hTERT*-immortalized HMECs indicative of a mechanistic tie between Ras activation and Myc expression? Previous studies of HMEC transformation have noted that amplification of c-*myc* occurred during the introduction of the SV40 early region, *hTERT*, and H-*ras*V12 into early passage HMECs (Elenbaas et al., 2001). Similarly, c-*myc* can replace H-*ras*V12 in the transformation of human fibroblasts in combination with *hTERT*, SV40 LT, and st (Wei et al., 2003). Since Ras signaling enhances the accumulation of c-Myc protein via inhibition of proteasome-mediated degradation (Sears et al., 2000), one consequence of Ras signaling is to elevate Myc levels.

The observation that overexpresssion of c-*myc* failed to replace H-*ras*V12 for HMEC tumor formation emphasizes the important role of Ras signaling in tumorigenesis. Ras transformation is mediated by activation of its multiple downstream effector pathways. Recent work has begun to elucidate the specific signaling pathways perturbed by H-Ras in the transformation of human cells. Using well-characterized Ras effector mutants, Hamad et al. reported that activation of PI3K was not essential to transform human fibroblasts, embryonic kidney epithelial cells, and astrocytes (Hamad et al., 2002). In these studies, activation of the RalGDS and Raf pathways played prominent roles in the transformation of human and murine cells, respectively. We believe these observations may be reconciled with the present observations since the effects of Ras on the PI3K pathway in the previous studies might have been masked by the presence of st. Moreover, a Ras effector mutant that only activated RalGDS permitted anchorage-independent growth, but failed to promote tumor formation (Hamad et al., 2002). This notion is supported by the observation that PI3K function was required for Ras transformation of NIH3T3 cells (Rodriguez-Viciana et al., 1997).

However, several observations indicate that H-*ras*V12-induced stimulation of the PI3K pathway fails to provide sufficient PI3K signaling for oncogenic transformation of human cells (Table 1) (Hahn et al., 2002). Since previous work has shown that high levels of H-*ras*V12 expression are required for tumor formation (Elenbaas et al., 2001), one possible explanation for these observations is that a threshold level of PI3K signaling is required for transformation, which cannot be provided by H-*ras*V12-induced signaling. An alternative explanation involves the spatial organization of activated signaling pathways. In the vicinity of an activated H-*ras*V12 molecule, multiple pathways are activated, perhaps in conflict with each other. In this case, expression of an activated allele of PI3K may provide the correct local environment for signaling or may counteract excessive Ras-induced signaling that might lead to growth arrest (Serrano et al., 1997). Although we still do not understand the relationships between Ras and PI3K signaling, experimental systems such as those described herein will provide important models for future studies.

The long-term cultivation of HMECs requires that such cells bypass several proliferative barriers. As several groups have reported, bypassing M0 appears to be dependent upon loss of expression of p16[INK4A] (Foster et al., 1998; Romanov et al., 2001), although conditions may exist that permit long-term cell growth without loss of p16[INK4A] (Herbert et al., 2002). Moreover, the further introduction of LT and *hTERT* permitted these HMECs to bypass replicative senescence. Since the upregulation of c-Myc appears to be a common feature of late passage *hTERT*-immortalized HMECs (Wang et al., 2000; Elenbaas et al., 2001), it remains possible that such late passage HMECs also harbor other genetic alterations. However, since the introduction of c-Myc into early passage HMECs recapitulated the transformation phenotypes of late passage HMECs (Figure 7), we concluded that it is unlikely that other cooperating genetic events in late passage HMECs contribute to the HMEC transformation observed here.

Although transformed cells that acquire the capability to grow in an anchorage-independent manner usually also form tumors in animal hosts (Cifone and Fidler, 1980), we found that some combinations of introduced genes confer only anchorage-

independent growth while others impart a full tumorigenic phenotype. Although the reasons for this difference in behavior remain unknown, these observations indicate that tumorigenic cells acquire one or more functional capabilities beyond that required for anchorage-independent growth. Identifying and understanding these additional steps will elucidate critical steps in cancer development.

In summary, we have identified the PI3K pathway as a critical signaling pathway targeted by st for the transformation of HMECs-hTERT. These observations have made it possible to create human cells whose transformation is critically dependent upon PI3K, Akt, and Rac expression and will allow us to construct more relevant models of human breast cancer. In addition, these experimental models will provide a useful platform for the testing and development of specific inhibitors of these oncogenic pathways.

Experimental procedures

Vectors and retrovirus production

The SV40 early region was introduced into the pWZL-blast retroviral vector (a gift from J. Morgenstern, Millennium Pharmaceuticals). cDNA versions of SV40 LT or st were cloned into pBabe-puro (Morgenstern and Land, 1990) and pWZL-blast, respectively. Myc-tagged Rac1V12 (Ridley et al., 1992) was cloned into pWZL-neo. pBabe-puro carrying human c-myc was kindly provided by M. Eilers, and this cDNA was also introduced into pBabe-zeo. The amino-terminal ends of p110α and Akt1 were modified by the pp60 c-Src myristoylation sequence (Klippel et al., 1996) and fused in frame with FLAG-epitope tag. Myr-FLAG-p110α and Myr-FLAG-Akt1 were cloned into pWZL-neo and pWZL-blast retroviral vectors, respectively. The p85ΔiSH2 (deletion of amino acids 478-513) (Dhand et al., 1994) cDNA was subcloned into pWZL-neo.

Amphotropic retroviruses were produced by transfection of the 293ε cells with packaging plasmids encoding VSV-G, gag-pol, and a retroviral vector encoding the gene of interest. Culture supernatants containing retrovirus were collected 48 hr posttransfection.

Cell culture

Early and late passage HMECs-hTERT (Clontech) were cultured in mammary epithelial basal medium (MEBM, BioWhittaker) supplemented with EGF, insulin, bovine pituitary extract, and hydrocortisone (termed mammary epithelial growth medium, MEGM) at 37°C and 5% CO_2 according to the manufacturer's instructions (BioWhittiker). BJ human foreskin fibroblasts were maintained in a 4:1 mixture of Dulbecco modified Eagle medium to M199 supplemented with 15% fetal calf serum. Stable cell lines were generated by serial infection of HMECs-hTERT or BJ cells with retrovirus carrying SV40 LT, st, or other specified genes. Cells were infected with viral supernatants in the presence of 4 μg/ml polybrene. After infection, successfully transduced polyclonal cell populations were obtained by selection with the appropriate drug (hygromycin [50 μg/ml], G418 [200 μg/ml], puromycin [0.5 μg/ml], blasticidin [2.5 μg/ml], or 500 μg/ml zeocin]. Infection frequencies were typically 20%–30%.

Immunoprecipitation and immunoblotting

Cells were lysed in 20 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40), 10% glycerol, 1 mM sodium vanadate, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. For assays requiring growth factor stimulation, HMECs were starved overnight in MEBM and treated with EGF (10 ng/ml) at 37°C for 10 min, or as indicated, and then lysed as described above. Lysates were centrifuged at 12,000 × g for 10 min at 4°C to remove insoluble material. For anti-FLAG immunoprecipitation, lysates were incubated with a M2-agarose affinity gel slurry (Sigma-Aldrich Co.) for 2 hr at 4°C. The immunoprecipitates were washed three times with lysis buffer, resuspended in Laemmli sample buffer, and boiled for 5 min.

Proteins from lysates (~60 μg of each) or immunoprecipitates were separated by 10% SDS-PAGE, then transferred to polyvinylidene fluoride membranes (Immobilon-P; Immobilon, Bedford, Massachusetts). Membranes were blocked and probed with the specified antibodies; namely, anti-phospho-Akt (Ser473 or Thr308), anti-Akt, anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-p44/42 MAPK (all from Cell Signaling Technology), anti-c-Myc (sc-764, Santa Cruz), anti-p85 (UB93-3, Upstate Biotechnology), anti-FLAG M2 (Sigma), anti-Actin (Sigma), anti-Rac (clone 23A8, Upstate Biotechnology), and PAb419 mAb specific for SV40 T antigens (Harlow et al., 1981) were used throughout.

Rac activation assay

Cells were grown until ~70% confluent, then starved overnight in MEBM. For the timecourse experiments, cells were treated with EGF (10 ng/ml) for the times indicated. Fresh cell lysates were prepared and subjected to Rac activation assay using Rac Activation Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions.

Growth curves

HMECs were plated at a density of 2.5 × 10⁴ per well in 12-well plates. At the indicated time points, cells were washed with PBS, fixed in 10% formalin, and rinsed with distilled H_2O. Cells were then stained with 0.1% crystal violet (Sigma) for 30 min, washed with distilled H_2O, and dried. Cell-associated dye was extracted with 2 ml of 10% acetic acid, and the optical density was measured at 590 nm. Values were normalized to the OD_{590} at day 0 for each cell type. Each point was determined in triplicate. For experiments performed with limited supplementation, MEBM was augmented with 0.5% of the standard growth factor concentrations.

Anchorage-independent growth assay

5 × 10⁴ HMECs were seeded per 60 mm plate with a bottom layer of 0.6% Bacto agar in DMEM and a top layer of 0.3% Bacto agar containing MEGM. Fresh MEGM (0.5 ml) was added after 1.5 weeks. Growth of BJ fibroblasts in soft agar was performed as previously described (Hahn et al., 1999). Colonies were scored after 3 weeks. Only those colonies with greater than 0.2 mm in diameter were counted. Such colonies are visible without microscopy and typically contain 50–60 cells. At least two independent assays were performed in triplicate.

Tumorigenicity assays

six- to eight-week-old immunocompromised mice (Cby.Cg-Foxn1ᵘ, Jackson Laboratory) were Υ-irradiated (400) rad prior to injection. HMECs cells (2 × 10⁶) resuspended in 100 μl of PBS were mixed with HMF cells (2 × 10⁶) resuspended in 100 μl of PBS and injected subcutaneously. For Matrigel (Becton Dickinson) injections, HMEC cells (4 × 10⁶) were resuspended in 100 μl of PBS and 100 μl of Matrigel. Tumor size was monitored every 5 days. Mice were sacrificed when the tumors reached a diameter of ~1 cm or after 16 weeks of monitoring.

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